A STUDY OF THE RELATIONSHIPS BETWEEN THE TWENTY-FOUR HOURLY URINARY OUTPUTS OF 17-KETOSTEROIDS AND CREATININE, AND THE WEIGHTS OF TWENTY ADULT MALE SUBJECTS FROM EACH OF SIX ETHNIC GROUPS

by J. W. H. LUGG AND J. M. BOWNESS

(From the Department of Biochemistry, University of Malaya, Singapore).

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An ethnological study of the type here presented might have, as its origins, several purposes which need not necessarily be in harmony. Lack of facilities prevented a practical start being made upon the work herein described until late in 1952. Its crude outline had been formulated, however, in mid-1951, as a result of repeated requests having been received for *ad hoc* estimations of the 17-ketosteroid urinary outputs of hospital patients, the large majority of whom being, in this locality (Singapore Island and the Malay Peninsula), of other than European stock.

Although some work had been done on Chinese subjects (Wu and Chou, 1937; Chou and Wang, 1939) the only substantial information available to us by 1952 about urinary excretion of 17-ketosteroids by human subjects pertained to those of European extraction and thus, probably, of essentially Caucasoid racial composition (see, for example, Fraser, Forbes, Albright, Sukowitch and Reifenstein, 1941; Nathanson, Towne and Aub, 1941; Wooster, 1943; Hamburger, 1948; Kenigsberg, Pearson and McGavack, 1949; and references given by Dorfman, 1948). It had long been recognised that mature, non-senile, healthy European men excreted greater quantities of 17-ketosteroids than did their female counterparts (apparently about one-and-a-half times as much: for review see Dorfman, 1948), the additional quantity in the case of the male being commonly attributed to the metabolism of testicular hormones (for a more recent example of expression of this view see Engel, 1954), the rest (as in the female) being attributed to the metabolism chiefly of hormones secreted by the adrenal cortex. There is now some doubt in our minds that the differences between adult male and adult female excretions can be accounted for satisfactorily in this way, but accepting the view then current and being cognizant of the apparent femininity of Chinese men (more especially, perhaps, those of the South) when compared with European men (for independent comment see Latourette, 1947, and Lin

Yu-tang, 1939), we felt that it would have been rash indeed to furnish for diagnostic purposes, especially in respect of suspected liver-damage, eunuchoid state, hirsutism, Addison's disease, etc., *ad hoc* estimates of the 17-ketosteroid outputs of persons other than Europeans.

From time to time evidence has emerged that the 17-ketosteroid output of normal subjects can be affected by a wide variety of factors in addition to sex and age. Among such other factors have been the conditions of rest, sleep, wakefulness and physical activity (Werner, 1941; Pincus, 1943; Dingemanse and Huis in't Veld, 1950), urinary volume (McHenry, Semmons, Pearse and Meyer, 1947; Neukomm and Reymond, 1950), emotional state of the subject (Pincus, 1943), climate (Wu and Chou, 1937; Robinson, Howard and Macfarlane, 1955). The validity of the evidence has not always gone undisputed, and counterclaims have sometimes been made by other investigators. The evidence available by 1954 for and against the effectiveness of these factors, has been briefly, but fairly comprehensively, reviewed by Higgin (1954).

Insofar as such factors may exert appreciable effects it is clearly necessary, when seeking to assess and compare the outputs of individual subjects and of groups, to collect the urine samples under standard conditions. The southern part of the Malay Peninsula and Singapore Island lie very close to the Equator and seasonal changes are therefore very small. Degrees of activity and rest are not easily standardised if subjects are living their normal lives, but by collecting urine samples over a 24-hour period the investigator does ensure, to some extent, that the samples pertain to a more or less standard, repetitive cycle of activity and rest. We proposed to accept these conditions, in the Malayan environment, as the standard conditions.

In acceptance of the view, then, that ethnic groups might differ in 17-ketosteroid excretion as well as in skin-colour and other such obviously variable characteristics, this investigation, designed originally to establish 'normal' ranges of values for the different ethnic groups accustomed to seek the aid of Western medicine (and confined, in the first instance to males), was almost axiomatically addressed likewise to the question whether, should the distributions of normal 17-ketosteroid excretion values prove to differ with the different ethnic groups, there might yet exist at least one common measure of "17-ketosteroid status" in normal adult (male) members of all ethnic groups. Therein, however, lay the possibility of incorporating conflicting aims in the one investigation, for such a measure might, of its very design, involve the mutual cancellation of pieces of evidence of abnormality in pathological cases.

Del Tufo (1949) may be consulted for more detailed information on the "national" composition of the polyglot population of Malaya. The Malays (commonly[1] regarded as being of predominantly Mongoloid composition) and Chinese (emanating mainly from the Southern provinces of China and regarded as essentially of Mongoloid composition) comprise the bulk of the population.

[1] Though not necessarily correctly—see Lugg and Whyte (1955).

A substantial Indian minority (emanating chiefly from Southern India, and regarded, perhaps, as of Caucasoid-"Carpentarian" composition — see Birdsell, 1950), is fairly numerous. Both Europeans (chiefly of recent English origin, and regarded as essentially Caucasoid in composition) and Eurasian (mainly of Portuguese-Southern Indian origin) constitute small minorities. Residues of aboriginal groups (Negrito, Sēnoi and Aboriginal Malay) persist in the Federation of Malaya. Of these groups, the Sēnoi appear to be of predominantly Mongoloid composition, but it is not possible, as yet, to describe the composition of the Negritos in terms of one or more of the currently-accepted main racial divisions (see Polunin, 1953; Sneath, 1953; Lugg, 1955, 1957).

With the object of obtaining information from highly diversified human material, the better to explore the various possibilities, the study was planned to embrace, with the four civilised groups of fairly long-established genic composition (*viz.* Southern Chinese, European, Tamil Indian and Malay) two uncivilised ethnic groups which rarely have contact with Western medicine, namely, the Sēmai branch of the Sēnoi and the Kintak Bong branch of the Negritos, both of which inhabit the jungles of the Malay Peninsula, and are believed (from their geographical locations and other evidence) to be of long stabilised genic composition.

Following the formulation of this programme of work there appeared an important article (Barnicot and Wolffson, 1952) dealing with the difference in excretion of 17-ketosteroids as between European and African adult male subjects. The results of this study, in view of the traditional "virility" of African males, constituted a serious puzzle to us because of the low values found for the African males. From military personnel who, during World War II, had had considerable association with bodies of troops belonging to various ethnic groups, we have since been given reason to believe, however, that the tradition is grossly ill-founded; but the work of Barnicot and Wolffson (1952) did confirm the suspicion that, for whatever reason, ethnic-group differences could be large.

Our work on Chinese, European and Malay subjects had been virtually completed early in 1954, and in a preliminary communication we (Lugg and Bowness, 1954) dealt briefly with the significance of the results. Since then we have completed our work on the Indian, Negrito and Sēnoi subjects. Meanwhile, Friedmann (1954) has shown for male subjects that the 17-ketosteroid excretions of Northern and Southern Indians are appreciably lower than those of Europeans. More recently we (Lugg and Bowness, 1955; Bowness, 1955) have given brief reports of the general nature of our findings for the six ethnic groups we have examined. It is the purpose of this article to present in appropriate detail, the work itself and the conclusions we are disposed to draw from it.

EXPERIMENTAL.

The studies of Wooster (1943), Hamburger (1948), Robinson (1948), and Birke and Plantin (1954) indicate that among normal, healthy, male Europeans,

the 24-hourly urinary excretion of 17-ketosteroids varies but little with age in the age-range 17-46 years, different authorities agreeing most closely in the conclusion that the excretion is perhaps maximal in the age-range, 20-40 years.

The subjects.

Our work was confined to apparently normal, healthy[1] male subjects aged 18 to 46 years, on the assumption that such (apparently) suitable age-limits for European subjects would be suitable also for other ethnic groups. The ages of aboriginal subjects had to be estimated by inspection, but the estimates are believed not to have been in error by more than ± 10 p.c. The first twenty acceptable subjects were taken from each group, care first being taken merely to ensure that the ages of the majority of subjects lay in the 20-40 year range.

The Chinese subjects were mainly University students. They were all of Southern Chinese provincial origin, though most of them were one or two generations removed from China and had been born in Malaya.

The European subjects were predominantly of English origin and most had been born in England. There were appreciable Irish, some Scottish and small Czech and Russian influences.

The Indian subjects were all Tamil and largely of immediate (or fairly recent) origin in the Madras Presidency in India, but an appreciable fraction was of immediate Ceylonese origin.

The Malay subjects were very largely of "Peninsula" origin and many had been born in the State of Malacca. Their progenitors had probably come, for the most part, from Sumatra to Singapore Island and thence to the Malay Peninsula proper during the 14th century (see Wilkinson, 1920).

The Kintak Bong division of the Negritos, during the first decade of the 20th century, appears to have been spread between the Western coast of the Malay Peninsula and the foothills of the central mountain chain on the East, Sēlama on the South and the Trik-Baling region on the North. Of this division (then several hundred strong) Schebesta (1927) found in 1924 that the remnants (61) which had survived a severe epidemic disease ("pneumonic" influenza?) had congregated in the Baling district. It was from these people and their descendents that our Negrito sample was drawn. From their headman's estimate of the present "pure" Kintak Bong population (*viz.* 78), it seems that the numbers have changed but little during the past 30 years.

The Sēmai division of the Sēnoi is the centrally placed one and therefore least likely to be subject to recent 'mixing' (Williams-Hunt, 1953; Noone, 1956).

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[1] Zubiran and Gomez-Mont (1953) refer to a lowering of 17-ketosteroid output during periods of undernourishment. There was sometimes observed among the Sēmai Sēnoi a tendency to parotid gland enlargement, which might possibly have been indicative of recovery from a period of undernourishment (see also Polunin, 1953), but the subjects were by no means emaciated at the time this work was done. Though small of stature they were mostly well-built and possessed of considerable strength and powers of endurance.

Our Sēnoi sample was drawn from a section of the Sēmai which, as part of the current "emergency" operations, had been concentrated from nearby districts into the Batang Padang district of Perak.

Collection and preservation of urine samples.

The subjects all lived the normal, everyday existence of their respective ethnic groups during the period (24 hours) of urine collection. They were given the usual instructions about complete emptying of the bladder just prior to and at the completion of the period and, in the case of aboriginal subjects, these two acts were supervised. They were exhorted to inform the collector about any loss of sample. The climate conditions for all subjects were very uniform — mildly warm or mild nights of moderately high humidity, and warm days ($28 \pm 2°$ C. shade temperature) of high humidity (approx. 80 p.c. rel. humid.).

Each sample was collected direct in a Winchester-quart bottle containing three drops of toluene, and was measured for volume and processed for transport and/or temporary storage at approx. 0° C., if it could not be assayed immediately. The processing consisted in adding 0·5 ml. of 5N hydrochloric acid to a 25 ml. portion of the urine (see Cameron and Guthrie, 1933) for creatinine assay, and of a mixture of equal weights of NaH_2PO_4 and $Na_2HPO_4.12H_2O$ (in the proportion of 1·0 gm. of mixture per 100 ml. portion of the urine) for 17-ketosteroid assay. This buffer mixture was employed because we observed that a urine sample so treated suffered a loss of "17-ketosteroids" of only 5·6 p.c. after 10 days' storage at 0° C., the pH changing from 6·1 to 7·2, and 13·5 p.c. loss after 10 days' storage at 28° C., the pH changing from 6·1 to 8·0. The indications are that changes undergone by the 17-ketosteroids are relatively small if the urine samples are kept cold and at pH values near those normally found with freshly voided urine (see Engel, 1954). Appropriate corrections were made for the small volume changes caused by the addition of the processing reagents.

Methods of estimation.

The Callow, Callow and Emmens (1938) modification of the Zimmermann (1936) colorimetric method, essentially as described by Warren (1950), was selected for the estimation of extracted urinary 17-ketosteroids, partly because of Warren's (1950) favourable appraisal of it and partly because of the desirability of having a means of comparing the findings of Barnicot and Wolffson (1952), who used this method, directly with those we hoped to obtain. The hydrolysis and extraction procedure recommended by Warren (1950) and based upon that of Robbie and Gibson (1943), was followed.

A "Spekker" photoelectric absorptiometer fitted with heat-absorbing Ilford filters (H697) was used for measuring the extinctions, firstly with light filtered through an Ilford 604 "spectrum"-green filter (yielding E_G), and secondly with

light filtered through an Ilford 601 "spectrum"-blue filter (yielding E_B).[1] A "reagent" blank (used for setting the instrumental zero) and an "extract-colour" blank were always prepared, and the E_G and E_B values were first adjusted for the extract-colour contributions which were sometimes very considerable, especially with Negrito material. The values so obtained, E'_G and E'_B, were substituted in the equation indicated by Talbot, Berman and MacLachlan's (1942) study to provide the final, corrected E_G value [E'_G corr. $= (E'_G - 0{\cdot}6E'_B)/0{\cdot}73$]; and from this and the E_G value of the standard the "17-ketosteroid" content of the extract was computed in terms of the standard substance (dehydro*iso*androsterone, *viz.* dehydroepiandrosterone). It should be noted that in the work which yielded the numerical values, 0·6 and 0·73, in the above equation, Talbot *et al.* (1942) used dehydro*iso*androsterone as standard 17-ketosteroid. The prepared urine extracts contained a variety of 17-ketosteroids possessing somewhat different chromogenic properties in the modified Zimmermann reaction (e.g. see Callow, Callow and Emmens, 1938). The proportions in which they occurred, however, appeared to be similar for all six ethnic groups involved in this study (see Appendix; also Bowness, 1955), and by "17-ketosteroid" content we mean this mixture of 17-ketosteroids estimated in the manner described above.

Urinary creatinine was estimated (by the method of Folin, 1914), partly to furnish a check upon the completeness of collection of the 24-hourly urine outputs, but also with the object of characterising the human material under investigation and (possibly) of helping to provide a means of measuring "17-ketosteroid status".

Inasmuch as it seemed reasonable to assume that 17-ketosteroid output might be related directly to a function of body-weight, and inasmuch as creatinine output is customarily considered to be correlated positively with body-weight (vide the so-called "creatinine coefficient"), it seemed desirable to estimate the body-weights of the subjects. Their weights, including clothing, were measured, usually by means of a spring-balance, and a correction for the clothing worn was applied to bring the weights to a "nude" basis.

RESULTS.

The basic data are given in Tables 1-6. Each of these tables is for one of the ethnic groups and shows, in the order of the columns, the subject's characteristic number, his age in years at last birthday, the volume of urine excreted in the 24 hr. in ml., the nude weight of the subject in Kg. (W), the creatinine excreted per 24 hr. in gm. (C) and the 17-ketosteroid excreted per 24 hr. in mg. (K). Certain derived statistics, namely, means, ranges and estimates of the "population" (*viz.* "group") standard deviation (S.D.) are also shown.

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[1] The 604 filter has a transmission peak at $\lambda = 520$ mμ, and the 601 filter (rather blue-violet in tint but referred to by some authors as a "violet" filter) has a peak at $\lambda = 430$ mμ.

TABLE 1.

Basic data for Chinese males.

Subject	Age in yr.	Urine Volume in ml.	Weight in Kg. (W)	Creatinine in gm./24 hr. (C)	17-Ketosteroids in mg./24 hr. (K)
C1	23	1240	42·9	1·75	12·2
C2	22	1275	48·3	1·50	10·6
C3	35	870	50·6	1·39	3·9
C4	20	850	48·3	1·22	9·0
C5	23	1275	59·2	1·81	17·5
C6	19	1020	58·8	1·14	5·4
C7	19	3000	58·9	1·26	6·8
C8	22	1800	63·0	1·91	14·8
C9	19	1310	54·2	1·39	10·9
C10	20	1990	60·1	1·75	6·5
C11	22	2175	68·2	1·87	8·5
C12	26	1680	48·3	1·46	6·5
C13	20	2330	50·5	1·62	8·7
C14	22	370	48·7	0·90	7·5
C15	21	2440	52·8	1·61	13·1
C16	24	1690	60·0	1·69	8·3
C17	23	580	48·9	1·36	9·6
C18	37	1640	40·1	1·28	6·6
C19	22	1370	52·8	1·21	10·4
C20	24	2360	50·5	0·94	8·0
Mean	23·2	1563	53·26	1·453	9·24
S.D.	4·78	678·7	6·980	0·2957	3·296
Range Min.	19	370	40·1	0·90	3·9
Range Max.	37	3000	68·2	1·91	17·5

TABLE 2.

Basic data for European males.

Subject	Age in yr.	Urine Volume in ml.	Weight in Kg. (W)	Creatinine in gm./24 hr. (C)	17-Ketosteroids in mg./24 hr. (K)
E1	24	1660	67·1	1·99	12·4
E2	45	2010	59·1	1·57	11·9
E3	31	1860	60·6	1·73	16·5
E4	22	1800	61·8	1·62	13·1
E5	39	1610	92·3	2·06	14·5
E6	30	1070	70·5	1·92	10·5
E7	38	1260	53·6	1·41	15·2
E8	27	1000	86·8	2·20	17·5
E9	26	1660	91·9	2·47	14·4
E10	34	2780	71·0	1·72	15·5
E11	32	1080	89·0	1·75	13·9
E12	26	1740	88·0	2·36	17·1
E13	46	980	70·9	2·12	11·7
E14	22	1500	85·2	1·53	21·6
E15	29	1940	73·0	2·29	27·9
E16	27	790	68·1	1·69	11·6
E17	31	1705	70·9	1·76	22·2
E18	35	1610	69·7	1·87	11·4
E19	35	2000	75·0	2·24	27·0
E20	38	740	65·9	1·75	13·6
Mean	31·8	1540	73·52	1·902	15·98
S.D.	6·92	498·7	11·554	0·3002	5·023
Range Min.	22	740	53·6	1·41	10·5
Range Max.	46	2780	92·3	2·47	27·9

TABLE 3.

Basic data for Indian males.

Subject	Age in yr.	Urine Volume in ml.	Weight in Kg. (W)	Creatinine in gm./24 hr. (C)	17-Ketosteroids in mg./24 hr. (K)
I1	19	750	62·0	1·25	10·2
I2	19	700	74·7	2·42	16·8
I3	22	1000	47·9	1·28	12·9
I4	22	1300	64·1	1·59	9·0
I5	24	1135	65·0	1·84	15·8
I6	20	630	49·0	1·02	5·4
I7	46	1580	63·2	1·34	7·4
I8	29	2870	58·3	1·44	8·6
I9	22	1295	42·9	1·19	9·0
I10	28	600	59·0	2·04	9·9
I11	21	790	46·6	1·46	8·8
I12	28	1250	49·2	1·77	11·4
I13	28	1000	59·7	1·70	10·0
I14	40	2380	56·0	1·43	8·6
I15	35	1125	56·0	1·34	8·4
I16	35	1210	48·9	2·31	10·4
I17	20	680	47·7	0·97	11·6
I18	21	1570	66·0	1·88	8·3
I19	20	1590	58·2	1·53	8·2
I20	34	2230	57·3	1·18	8·6
Mean	26·6	1284	56·58	1·549	9·96
S.D.	7·76	616·5	8·117	0·3980	2·704
Range Min.	19	600	42·9	0·97	5·4
Range Max.	46	2870	74·7	2·42	16·8

TABLE 4.

Basic data for Malay males.

Subject	Age in yr.	Urine Volume in ml.	Weight in Kg. (W)	Creatinine in gm./24 hr. (C)	17-Ketosteroids in mg./24 hr. (K)
M1	26	1450	52·0	1·22	8·5
M2	25	1270	54·2	1·32	8·3
M3	24	740	52·9	1·47	6·3
M4	22	600	50·7	1·41	8·8
M5	21	800	54·2	1·51	14·9
M6	35	740	57·5	1·70	6·6
M7	36	780	67·4	1·76	7·2
M8	30	700	54·2	1·49	6·0
M9	43	800	68·3	1·83	10·2
M10	43	710	49·7	1·41	5·7
M11	39	1535	62·9	1·49	8·4
M12	30	820	51·9	0·93	4·4
M13	32	760	61·0	1·51	5·2
M14	30	355	51·8	0·85	4·7
M15	33	920	51·8	1·13	5·5
M16	22	1134	59·9	1·42	14·4
M17	21	780	56·3	1·40	9·3
M18	24	1480	68·2	1·72	9·9
M19	27	1180	63·7	1·27	10·9
M20	37	1450	72·7	1·88	8·9
Mean	30·0	950	58·06	1·436	8·20
S.D.	7·09	336·7	6·990	0·2723	2·911
Range Min.	21	355	49·7	0·85	4·4
Range Max.	43	1535	72·7	1·88	14·9

TABLE 5.

Basic data for Negrito males.

Subject	Age in yr.	Urine Volume in ml.	Weight in Kg. (W)	Creatinine in gm./24 hr. (C)	17-Ketosteroids in mg./24 hr. (K)
N1	20	930	44·8	0·97	3·5
N2	22	558	43·7	1·48	5·6
N3	33	1156	41·5	0·55	2·6
N4	25	1106	43·7	1·37	3·4
N5	20	664	41·6	1·15	4·3
N6	20	496	37·5	0·74	2·7
N7	34	194	41·6	0·40	1·8
N8	19	799	34·3	1·09	5·2
N9	18	382	34·9	0·39	1·4
N10	25	498	42·0	1·13	4·8
N11	35	390	37·9	0·85	6·2
N12	30	829	51·1	1·37	6·9
N13	43	279	43·8	0·95	4·3
N14	25	402	42·5	0·84	4·7
N15	20	1098	38·9	1·65	8·2
N16	35	302	43·0	0·83	5·6
N17	19	815	38·8	1·02	6·0
N18	19	393	40·2	1·22	4·8
N19	19	615	45·2	1·63	5·4
N20	32	1110	42·0	1·18	5·8
Mean	25·6	651	41·45	1·040	4·66
S.D.	7·39	310·0	3·808	0·3629	1·713
Range Min.	18	194	34·3	0·39	1·4
Range Max.	43	1156	51·1	1·65	8·2

TABLE 6.

Basic data for Sēnoi males.

Subject	Age in yr.	Urine Volume in ml.	Weight in Kg. (W)	Creatinine in gm./24 hr. (C)	17-Ketosteroids in mg./24 hr. (K)
S1	28	787	53·5	1·56	9·8
S2	25	397	63·5	1·44	4·3
S3	30	320	48·9	1·31	6·6
S4	20	463	45·8	0·71	3·1
S5	40	490	52·1	0·95	4·3
S6	45	594	54·9	1·51	7·2
S7	25	412	41·7	0·76	3·8
S8	30	589	51·2	1·15	3·4
S9	36	980	42·6	0·92	7·0
S10	26	592	44·4	1·21	4·1
S11	25	783	48·5	1·13	4·7
S12	43	693	44·9	1·07	4·0
S13	38	795	42·5	1·43	7·7
S14	44	548	66·6	1·35	3·8
S15	25	559	56·6	1·02	3·7
S16	35	372	41·6	0·80	6·2
S17	18	278	43·5	0·69	4·0
S18	37	622	43·5	1·19	5·6
S19	20	1215	52·5	1·31	5·8
S20	25	1012	51·1	1·30	5·6
Mean	30·8	625	49·50	1·140	5·24
S.D.	8·40	245·1	7·105	0·2689	1·748
Range Min.	18	278	41·6	0·69	3·1
Range Max.	45	1215	66·6	1·56	9·8

It must be pointed out, in passing, that whilst the Kintak Bong Negrito sample comprised about three-quarters of the adult male population in the age-range 17-46 years, we have adopted the view (see also Lugg, 1957) that it should be regarded as a small sample of a potentially large (over the generations, anyway) 'population'. The usual statistical formulae apply under such conditions. At the same time, there are certain peculiarities about the Negrito material which bring the adequacy of such an approach into question. The matter is referred to in the Discussion.

TABLE 7.

Twenty-four hourly urine volumes and 17-ketosteroid outputs of four subjects, each on two different occasions (a and b), the weights of the subjects remaining virtually unchanged.

Subject	Period between collections (months)	24-hourly urine vol. (ml.)	K
C19	3·3	(a) 1370 (b) 560	(a) 10·4 (b) 10·4
C20	3·3	(a) 925 (b) 2360	(a) 8·0 (b) 7·1
E2	1·8	(a) 2010 (b) 2860	(a) 11·9 (b) 12·6
E17	25·2	(a) 1705 (b) 2690	(a) 22·2 (b) 21·7

In Table 7 are given the results of repeat tests with four different subjects, the results (for the same subjects) used in compiling Tables 1 and 2 being shown for comparison.

DISCUSSION.

Examination of Table 7 suggests that the reproducibility of estimates of 17-ketosteroid output obtained under our conditions of collection and working is eminently satisfactory. It is also of importance to observe that the urinary volume appears to have little, if any, effect *per se* upon the 17-ketosteroid output, a finding that is in conformity with the conclusions of Wooster (1943), and of Neukomm and Reymond (1950). It might be mentioned, in passing, that a brief study (the data are not given here) of the rates of 17-ketosteroid excretion of subjects E1 and E2 at different times of the day indicated, in conformity with the findings of Pincus (1943), that periods of wakefulness and physical activity are associated with somewhat greater rates of excretion than are periods of sleep and rest.

The age means and measures of scatter shown in Tables 1-6 serve merely to indicate the general age composition of the samples and do not purport to repre-

sent the distributions of ages (within the 17-46 yr. range) among the males in the ethnic groups themselves. The urinary volume (for the 24 hours) is, to some degree, a reflection of the liquid-intake habit of the subject. This, in turn, may be conditioned by social circumstances—e.g. primitively-living aborigines often have less easy access to potable liquid than have most civilised people. Other things being equal, a larger subject with, presumably, a larger amount of excretory products to discharge, might be expected to employ a correspondingly larger amount of liquid for the purpose, and the urinary volume might be expected to reflect, at least in some measure, the mass of the subject. The individual volumes, the means, and the measures of scatter, and the correlations between the group mean urinary volumes on the one hand and the group mean W (or C or K) values on the other, such as may be apparent from a glance at Tables 1-6, are to be viewed in the light of these comments.

It is believed that the other variates, namely, weight, creatinine excretion, and 17-ketosteroid excretion, reflect (substantially under random-sampling conditions) the corresponding distributions for normal male subjects of age 17-46 years in the ethnic groups themselves. The usefulness of the derived statistics pertaining to these variates is, in appreciable measure, determined by the degree to which the distributions of the values approximate to 'normal'. So few values (twenty) are involved for each variate of each ethnic-group sample that a traditional-type histogram or frequency polygon cannot be expected to reveal at all clearly the nature of the distribution—even a small change in class-mark values (the class-span remaining unchanged) can have a marked effect.

As a means of smoothing out to some extent the chance irregularities and thereby elucidating the general nature of frequency distributions, Lugg and Whyte (1955), in a different type of study published from this laboratory, have employed class single-values instead of class-spans and have divided each "unit" observation between two contiguous classes in the proportion indicated by the value of the observation and the values of the contiguous classes between which it lies. The procedure has certain defects, some of which are common also to the traditional procedure, but it has the advantages of reducing the effects of chance irregularities and of permitting computation of the exact arithmetic mean from the grouped data (the variances are ordinarily only slightly larger than are those computed from the individual values).

Fig. 1 shows, respectively for the W, C and K values (from left to right), the families of frequency polygons (one polygon for each ethnic group) obtained by employing this same procedure (the classes being valued as indicated and having no span) with the data in Tables 1-6. The tails of the polygons have been produced to meet the abscissae at the class values next beyond the limiting classes containing material. Associated with each polygon are three points indicated by the centres of two small circles (representing the points of inflexion of the 'normal' curve fitted to the grouped data) and a disc (repre-

senting the peak of the fitted normal curve). These points thus also show diagrammatically, and without obscuring much of the polygon, the position of the mean and the magnitude of the standard deviation of the grouped data. For none of the variates does there appear to be definite evidence of departure of the 'population' distributions from mesokurtosis. It will be observed that it is only in respect of the K values that the suggestion of skewness in the distributions is sufficiently marked and consistent in direction (a positive skewness is discernible) to justify the view that a transformation of the variate should be attempted in an endeavour to render the distributions substantially symmetrical.

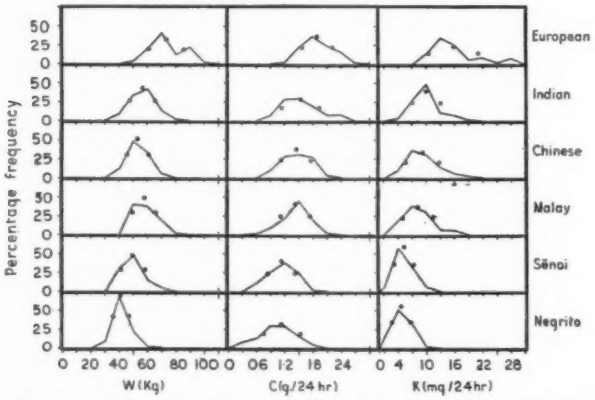

Fig. 1. Frequency distributions of values of W, C and K in each of the six ethnic group samples.

A cursory examination of Tables 1-6 suggests that the sample mean creatinine excretion tends to vary directly and linearly with the sample mean weight. On the other hand, it appears that the sample mean 17-ketosteroid excretion (apart from small aberrations) might vary directly and linearly, not with the sample mean weight or the sample mean creatinine excretion, but with a power thereof, the power exceeding unity. The fact that the sample mean K varies so much more rapidly with sample mean W than does sample mean C poses some questions. The sample mean W itself varies so greatly that it might be conjectured that in respect of adult hormonal controls as well as of those operating embryonically and in infancy, there might be ethnic-group differences, and that these might be reflected in changes in the proportions of the different 17-ketosteroids appearing in the urines. From fractionation experiments described in the Appendix to this article (see later) we have been obliged to conclude that the proportions were substantially the same for all the ethnic groups.

In examining in greater detail the natures of the distributions of the simple variates and the relationships between these variates and/or transformations

of them, we have made an important tentative assumption. This assumption is that humanity as a whole (or at least the 17-46-year-old male part of it) possesses some attributes or qualities of which the appropriate measures are actually (or potentially) 'normally' distributed. On the basis of this assumption the mean value of W, C or K for each sample has been plotted (the diagrams are not reproduced here) against the corresponding estimate of 'population' (i.e. group) variance and standard deviation, as a possible means of obtaining information about the nature of the distribution of that form of the variate in the envisaged 'humanity'. It is thus found that the individual sample estimates of 'population' standard deviations of the C values may be regarded as disposed about a straight line lying roughly parallel with the axis of sample-mean C values; and the presumption is that the C values in this 'humanity' are normally distributed. The sample estimates of 'population' standard deviations of the K values lie closely about a straight line passing through the origin in the co-ordinate system relating the sample means of K values with the corresponding estimates of 'population' (or group) standard deviations; and the presumption is that the K values in this 'humanity' approximate to being 'log-normally' (see Gaddum, 1945) distributed. It may be mentioned in passing that a similar type of relationship was encountered by Robinson (1948) in samples of European males drawn from widely differing age-groups, but he did not draw therefrom any conclusion about the nature of the distributions. The sample means of the W values appear to be so related (if they are regularly related at all) to the sample estimates of 'population' variance or standard deviation as to suggest that in respect of the attribute, weight, the different ethnic groups may not constitute sections of a common humanity at all (Wootton, 1955), whatever the nature of the distribution of this variate in each ethnic group might be.

The implications contained in the last paragraph clearly do not, in any sense, preclude the possibility of there being correlations between the variates within each of the ethnic groups; but yet neither do they preclude it in all subjects taken all together, and inasmuch as the number involved is six times as large and the value ranges are much greater, the overall correlations might well be more pronounced than the group correlations. Figs. 2, 3 and 4 show, respectively, the scatter diagrams of W and C, W and K, and C and K for all subjects. In regard to Fig. 2 there would appear to be little to support any view other than that the regressions of W with C, one upon the other are rectilinear. But in regard to Figs. 3 and 4, the conclusion cannot be escaped that the regressions of W and K, and likewise of C with K, are curvilinear.

The broad relationship between the individual W and C values thus seems to reinforce what was earlier gauged from cross-comparison of the sample means of W and C values; and the same may be said of the relationships and comparisons of W and K values on the one hand and of C and K values on the other.

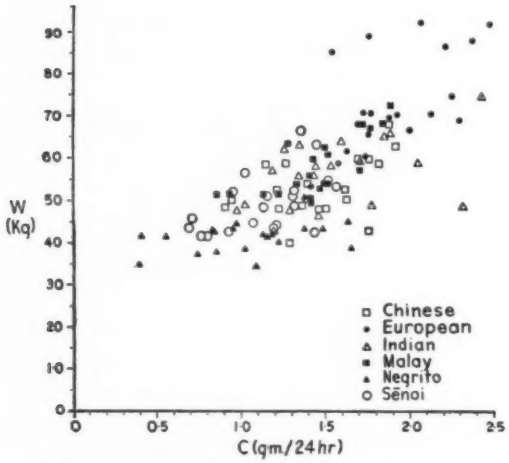

Fig. 2. Relationship between values of W and C.

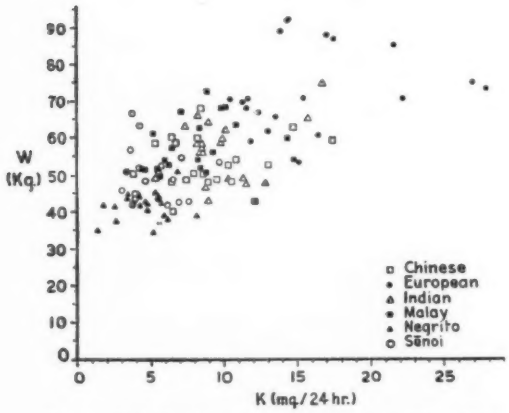

Fig. 3. Relationship between values of W and K.

Furthermore, it is found that if the simple transformation of the variate, K, into log K,[1] suggested by the results (referred to earlier) of plotting the mean values against the sample estimates of 'population' standard deviation, is made, the regressions of W and of C, respectively, with this transformation of K are substantially rectilinear.

[1] The term "log" is used for logarithm to the base 10 throughout this paper.

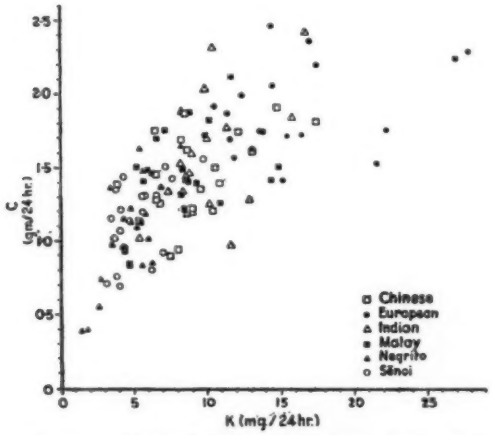

Fig. 4. Relationship between values of C and K.

We have earlier (Lugg and Bowness, 1954) shown that the sample mean K and log K values for some of these ethnic groups differed at high levels of statistical significance; and analyses of variance of the complete data now available show that in respect of C and W values as well as of log K values, the samples differ greatly (for W, $F = 37 \cdot 85$; for C, $F = 18 \cdot 48$; for log K, $F = 37 \cdot 38$. In all cases the corresponding probabilities are vanishingly small).

Correlations between urinary 17-ketosteroid and creatinine outputs (Nathanson *et al.*, 1941) and between urinary 17-ketosteroid excretion and weight (Wooster, 1943; Hamilton and Hamilton, 1948) have been found; but the significance of the correlations with weight has been disputed (see Higgin, 1954; and Fraser *et al.*, 1941). In a general way one might argue, on physiological grounds, that as W becomes smaller and smaller so too should C and K, whatever the precise nature of the relationships between them all might be. In this connection the transformation of K into log K presents undesirable characteristics when values of K become very small, and already with the Sēnoi and Negritos (especially the latter) some of the K values are small enough to suggest that a more satisfactory transformation might be sought. We have therefore tested also certain other transformations, namely, log $(2K + 1)$, log $(K + 1)$, and log $(K^2 + 1)$, which, unlike log K, tend to zero as K itself tends to zero, but which like log K, afford reasonably symmetrical distributions and reasonably linear regressions with W and with C.

In Table 8 are shown the correlation coefficients (r) of the variates, W, C and log K [or log $(K + 1)$, log $(2K + 1)$, or log $(K^2 + 1)$], taken in pairs, for each of the ethnic groups in turn and finally for all subjects together. The probabilities (P) of greater values of r arising by chance in sampling from an infinite "population" of uncorrelated pairs of variates, are also shown.

TABLE 8.

Correlation coefficients (r) with the corresponding probabilities (P) for some pairs of variates.*

Variates		Ethnic Group						Overall
		Chinese	European	Indian	Malay	Negrito	Sēnoi	
W and C	r	0·330	0·604	0·481	0·721	0·389	0·548	0·732
	P	>0·1	0·006	0·034	<0·001	0·088	0·013	≪0·001
W and log. K	r	0·119	0·236	0·265	0·391	0·285	0·124	0·651
	P	>0·1	>0·1	>0·1	0·087	>0·1	>0·1	≪0·001
W and log. (K+1)	r	0·123	0·234	0·266	0·385	0·270	0·122	0·658
	P	>0·1	>0·1	>0·1	0·090	>0·1	>0·1	≪0·001
W and log. (2K+1)	r	0·121	0·235	0·262	0·388	0·276	0·123	0·656
	P	>0·1	>0·1	>0·1	0·089	>0·1	>0·1	≪0·001
W and log. (K^2+1)	r	0·118	0·234	0·276	0·389	0·268	0·124	0·654
	P	>0·1	>0·1	>0·1	0·088	>0·1	>0·1	≪0·001
C and log. K	r	0·452	0·263	0·507	0·430	0·758	0·539	0·758
	P	0·046	>0·1	0·023	0·060	≪0·001	0·014	≪0·001
C and log. (K+1)	r	0·460	0·264	0·508	0·420	0·755	0·539	0·755
	P	0·042	>0·1	0·023	0·065	≪0·001	0·014	≪0·001
C and log. (2K+1)	r	0·456	0·264	0·508	0·425	0·756	0·539	0·757
	P	0·044	>0·1	0·023	0·063	≪0·001	0·014	≪0·001
C and log. (K^2+1)	r	0·454	0·262	0·507	0·427	0·756	0·539	0·757
	P	0·045	>0·1	0·023	0·062	≪0·001	0·014	≪0·001

* Values derived from Table VI of Fisher & Yates (1953).

An appreciable variability is to be discerned among the group values of each of the correlation coefficients and some of them are of very low statistical significance. The values of the overall correlation coefficients, however, are uniformly fairly large, and of extremely high statistical significance.

In respect of creatinine excretion it is clear that a useful measure of the 'status' is furnished by the compound variate, C/W. In respect of 17-ketosteroid excretion it is apparent that any measure of 'status' should have W or C (or perhaps both) in the denominator. Depending upon whether W or C is used in the denominator, the numerator requires to be changed to afford the best uniformity of measure, but the form of numerator which, in conjunction with either W or C as denominator, furnishes the most uniform measure and, at the same time, complies with the requirements mentioned in the third-to-last paragraph, appears to be log (K+1). As C and K tend to rise and fall together there is the possibility that, for any given subject, K/C might be fairly constant, even for short-period collections of urine samples. Among different subjects, C tends to vary with W, and it has already been indicated that K tends

to vary with a power (exceeding unity) of W or of C. These considerations suggest that the variate, K/(CW), might be sufficiently uniform to afford a useful measure of 17-ketosteroid "status" in different subjects even when the urine sample available is known (or believed) to pertain to other than a 24 hr. period of collection.

TABLE 9.

Means and measures of scatter of the compound variates for the ethnic groups taken separately and all together.

Compound variate		Ethnic Group						Overall
		Chinese	European	Indian	Malay	Negrito	Sēnoi	
C/W	Mean	0·0275	0·0261	0·0275	0·0247	0·0250	0·0231	0·0256
	S.D.	0·00543	0·00331	0·00659	0·00349	0·00836	0·00478	0·00571
	Range Min.	0·0185	0·0180	0·0202	0·0164	0·0096	0·0155	0·0096
	Range Max.	0·0408	0·0314	0·0472	0·0296	0·0424	0·0336	0·0472
log. (K+1)/W	Mean	0·0189	0·0168	0·0185	0·0164	0·0179	0·0161	0·0174
	S.D.	0·00334	0·00271	0·00288	0·00237	0·00397	0·00328	0·00325
	Range Min.	0·0136	0·0129	0·0146	0·0130	0·0108	0·0102	0·0102
	Range Max.	0·0261	0·0226	0·0239	0·0222	0·0248	0·0222	0·0261
log. (K+1)/C	Mean	0·703	0·651	0·697	0·675	0·758	0·714	0·700
	S.D.	0·148	0·108	0·159	0·123	0·188	0·165	0·151
	Range Min.	0·497	0·481	0·458	0·518	0·470	0·503	0·458
	Range Max.	1·033	0·885	1·134	0·889	1·118	1·072	1·134
K/(CW)	Mean	0·123	0·119	0·121	0·101	0·111	0·0984	0·112
	S.D.	0·0395	0·0407	0·0451	0·0348	0·0316	0·0379	0·0389
	Range Min.	0·055	0·063	0·067	0·056	0·057	0·042	0·042
	Range Max.	0·171	0·201	0·251	0·182	0·192	0·186	0·251

In Table 9 are shown the mean values, respectively, of C/W, log (K + 1)/W, log (K + 1)/C and K/(CW), for the various ethnic groups, taken singly and all together. The estimates of standard deviations and the ranges are also shown, but the individual values themselves have not been included.

In the first place, the high degree of uniformity of the mean values of any of these compound variates among the ethnic groups, is to be observed. The estimates of group standard deviations for any of the variates are less regular but, for the most part reveal appreciable uniformity.

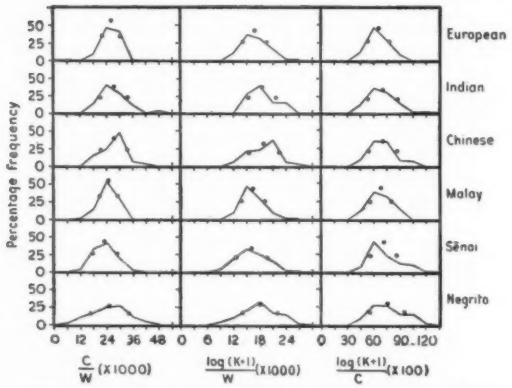

Fig. 5. Frequency distributions of values of some compound variates in each of the six ethnic group samples.

Fig. 5 shows, respectively, for the C/W, log $(K+1)/W$ and log $(K+1)/C$ values (from left to right) the families of frequency polygons obtained by dividing observations between contiguous classes and summing in the classes, as was done earlier with the W, C and K values. Here again the peaks and points of inflexion of the 'normal' curves fitted to the grouped data are indicated by the centres of the discs and of the open circles, respectively.

With these compound variates, as with W, C and K, there appears to be no definite departure from mesokurtosis in the "populations". With log $(K+1)/C$, only, is there skewness (positive in type) marked enough and consistent enough to suggest that it may well be a feature of the distribution of this variate in ethnic groups, but if it is a feature it is by no means a pronounced one. The nature of the distribution of $K/(CW)$ in the different ethnic groups has not been examined closely. Traditional-type histograms constructed from the individual values of this variate in each sample reveal no consistent trends as regards skewness, and one's first impression, therefore, is that its distribution in each ethnic group is likely to approximate to normal.

It is upon values of C/W, log $(K+1)/W$ and log $(K+1)/C$ that one would tend to rely for diagnostic purposes, as each involves only two variates, each of which in turn might be expected (other things being equal), on physiological grounds, to vary with the other. But a given subject might (for instance) possess an abnormally high log $(K+1)/W$ value and an abnormally low log $(K+1)/C$ value, and yet a $K/(CW)$ value which could only be classed as physiologically normal. Therein lies a weakness of more complex measures of status, such as $K/(CW)$, especially as recourse would normally be had to it under conditions (i.e. incomplete 24 hr. collection) which would not yield a value of C/W to convey a warning.

There can be little doubt that the appropriate idea of what constitutes the normal status of a subject is the mean value of the variate concerned, in the ethnic group to which he belongs, but we have examined only six ethnic groups. One of the criteria by which we were prepared to judge the usefulness of any compound variate was the uniformity of the ethnic group means and variances of that variate, and the question now arises whether the mean of the ethnic group means themselves (this is the same as the overall mean, in our work) would furnish a satisfactory idea of what might be regarded as normal status in a subject of some other, different, ethnic derivation.

Analyses of variances of the compound variates between and within groups, yield: for C/W, $F = 1{\cdot}89$, $P = 0{\cdot}10$; for log $(K+1)/W$, $F = 2{\cdot}76$, $P = 0{\cdot}021$; for log $(K+1)/C$, $F = 1{\cdot}15$, $P =$ c. $0{\cdot}35$; for $K/(CW)$, $F = 1{\cdot}51$, $P = 0{\cdot}19$. For all but one of these variates the values of the probability, P, are such as to indicate that certainly for subjects of other ethnic derivation and even indeed for subjects belonging to the ethnic groups we have examined, the overall mean might be expected to furnish an adequate idea of normal status. The exception is the case of log $(K+1)/W$, the value of P being small enough to indicate that the separate means should be used where applicable. The overall mean for each of these compound variates together with the estimate of overall standard deviation are shown in the final column of Table 8.

In view of our comments (below) on the Negrito material, the inclusion or omission of it in deriving overall means and measures of scatter should depend upon whether, in thinking of a generality of ethnic groups, one includes (as we have done) such small isolates as the Kintak Bong Negritos. From the individual ethnic-group means and estimates of standard deviation and from the overall means and estimates of overall standard deviation, on the assumption that the distributions of the variates are either 'normal' or approximate closely enough thereto, appropriate ± 10 p.c. and ± 1 p.c. points can readily be computed for use in the manner described by Wootton, King and Smith (1951) as an aid in detecting abnormality if present in a subject.

The possibility of some of our supposedly normal variates being so extreme as to warrant exclusion should be considered here. There is always the possibility of a urine sample having been incorrectly collected and then, too, subclinical conditions of abnormality could escape detection; and so, on grounds disclosed by statistical tests (extreme values of W, C or K, or of one of the compound variates) but not themselves of purely statistical nature, the inclusion of several subjects (in particular, perhaps C1, I16, N7, N9) might be brought into question.

We provide in Table 10 data from which replacement of some of the subjects might be made by those who feel that such replacement is desirable. There are risks in excluding (Irwin, 1955), and we ourselves have not been prepared to take them. Rather do we consider it safer, for example, to assume

TABLE 10.

Data from which some extreme variates may be replaced.

Ethnic group	Age (yr.)	24-hourly urinary volume (ml.)	W	C	K
Chinese	26	1395	56·1	1·53	9·8
Indian	29	2250	46·6	1·41	10·9
Negrito	19	338	41·1	0·78	2·0
	17	334	33·1	0·71	2·4

that if the somewhat larger variabilities of most of the compound variates in the Negrito sample are not due to fortuitously large sampling errors they may reflect a necessary condition for survival in a small ethnic group obliged to contend (even in the fairly benign Malayan jungle) with ever-changing, stressful circumstances.

CONCLUSION.

We are of the opinion that the success of our search for one or more common measures of 17-ketosteroid "status" justifies our view that for at least some purposes (including those we had in mind when commencing this work) ethnic group differences in 17-ketosteroid excretion by male subjects, like those in creatinine excretion, are conveniently regarded as reflections of the differences in weight. As by doing this we regard the weight as the independent variable, we are obliged to remember that administration of androgenic material can bring about an increase in muscle mass (Wilkins and Fleischmann, 1946).

The almost linear relationship between W on the one hand and log K or log $(K+1)$ on the other, suggests some sort of analogy with the well-known concentration-action relationship of Weber. Indeed, by regarding K as a measure of regulating hormones and modifying the approach in a general way, the analogy can be pushed quite far; but the real significance of the relationship remains obscure. No peculiarities in "status" with respect to 17-ketosteroids or creatinine accompany the somewhat feminine characteristics associated with certain non-Caucasoid male subjects.

SUMMARY.

Estimates of the weight in kilograms (W), and of the 24-hourly urinary outputs of creatinine in grams (C) and of 17-ketosteroids in milligrams (K), for twenty adult male subjects from the Chinese, European, Indian, Malay, Negrito and Sēnoi ethnic groups resident in Singapore and the Federation of Malaya have been obtained.

Of the variates W, C and K, the first two appear to be approximately normally distributed within the groups, whereas K appears to be approximately log-normally distributed.

Examination of the correlations (within ethnic groups and overall) between pairs of variates, suggests that values of the compound variates log $(K+1)/W$ and log $(K+1)/C$ might be of considerable use in assessing the 'status' of a subject in respect of his 17-ketosteroid output, just as C/W (a measure of the so-called 'creatinine coefficient') is of use in assessing the normality or otherwise of the 24-hourly creatinine output. Values of the compound variate K/CW may have a special use.

Analyses of variance have shown, for each of the variates W, C and log K, an extremely low probability that the samples from the six ethnic groups could all have been drawn from the same, normally distributed, population. The corresponding probabilities for the compound variates log $(K+1)/C$, $K/(CW)$ and, to a lesser extent, log $(K+1)/W$, for the same samples, are very much higher.

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APPENDIX

CHROMATOGRAPHY ON ALUMINA OF 17-KETOSTEROID MATERIAL FROM EACH OF THE SIX ETHNIC GROUPS

by J. M. Bowness

In view of the findings described in the main paper it seemed necessary to have some indication of whether there were differences in the relative amounts of individual 17-ketosteroids in the urines of the six ethnic groups studied. In the absence of any evidence to the contrary, the possibility existed that the differences which had been observed in the amounts of total 17-ketosteroid material excreted might be associated with differences in the proportions of the various 17-ketosteroid compounds.

To obtain information about the relative amounts of individual 17-ketosteroids excreted, it was decided to study the distribution of 17-ketosteroid material in the various fractions obtained by chromatographing material from each of the six ethnic groups on alumina, using the procedure of Dingemanse, Huis in't Veld and de Laat (1946), as given by Robinson and Goulden (1949). A preliminary report of this work has already been given (Bowness, 1955).

Materials and Methods.

In the estimation (Warren, 1950) of the total daily 17-ketosteroid excretion of individual subjects of the six ethnic groups, 3·6 ml. of the ethanolic solution of the material extracted from 100 ml. of urine by carbon tetrachloride was unused in the Zimmermann colour reaction. This volume of ethanolic solution from each of the 20 individuals of one ethnic group was combined with the other samples of that group and stored dry in a glass-stoppered flask at 4° C. After each addition of ethanolic solution to the flask the ethanol was evaporated on a water bath under filter-pump vacuum. Mixed extracts of urine of this type were obtained from each of the groups, Chinese, European, Indian, Malay, Negrito, Sēnoi. Some of the mixed extracts contained a few samples from subjects not listed for their total 17-ketosteroid excretion.

Before chromatography, each mixed extract was dissolved in ethanol and an aliquot portion assayed for total 17-ketosteroid content. A portion of the ethanolic solution was then removed and evaporated to dryness under reduced pressure, using benzene to remove the last traces of ethanol. The portions taken from the mixed extracts of each ethnic group contained roughly equal quantities of 17-ketosteroid material. The ethanol-free material from each mixed extract was dissolved in dry thiophen-free benzene (50 ml.) and chromatographed on Merck alumina (15 gm.) according to the procedure of Robinson and Goulden (1949). Forty-three eluate portions of 50 ml. each were collected by means of an automatic fraction collector. All traces of solvent were removed on a water bath using filter-pump vacuum. The dry residues of the eluate portions were dissolved in ethanol (1 or 2 ml. according to the amount of material present) and the 17-ketosteroid content of the ethanolic solutions estimated by the Zimmermann colour reaction, as given by Warren (1950), using also the correction procedure derived from the work of Talbot, Berman and MacLachlan (1942).

RESULTS.

The results of the estimations on the various eluate portions are presented graphically in Fig. 1. It can be seen in this figure that each of the mixed extracts has been separated into a number of 17-ketosteroid-containing fractions by the chromatographic procedure, each fraction being spread over a number of eluate portions.

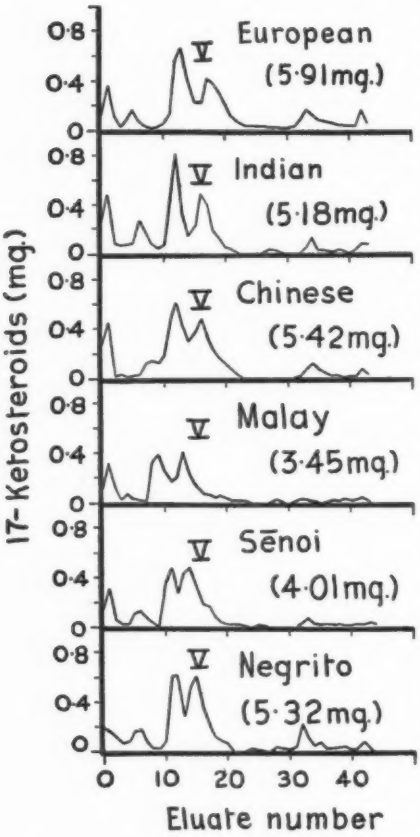

Fig. 1. Fractionation of 17-ketosteroid material from each of the six ethnic groups.

Most of the fractions which have previously been described and identified [see Robinson (1954)] are clearly evident in the distribution patterns obtained from each of the mixed extracts. Adopting the usage of Robinson (1954) as to the numbering (I-VIII, from left to right) and the contents of the fractions, peaks are evident in each of the patterns of Fig. 1 for fractions I (containing chlorodehydro*iso*androsterone), III (containing dehydro*iso*androsterone and *iso*androsterone), IV (androsterone and a little △-9 androstenolone), V (aetiocholanone, and a little △-9-aetiocholenolone), VII and VIII (both containing unidentified compounds, possible artefacts). Fractions II (containing androstandione and etiocholandione) and VI (containing 11-keto and 11-hydroxyetiocholanone) are not clearly evident in all the patterns, and their position and magnitude in some patterns can only be determined by analogy with patterns in which they are obviously present, and by interpolation from the peaks on either side.

The peaks of the various fractions in the distribution patterns of Fig. 1 correspond roughly in their relative positions and magnitudes with those obtained by Robinson and Goulden (1949) for normal male European subjects, though it is necessary to take into account the total amount of material placed on the column and the amount of material in the previously eluted fractions in

TABLE 1.

Percentage composition of chromatographed 17-ketosteroid material from each of six ethnic groups.

Fraction no.	Percentages of total eluate 17-ketosteroid material in the eight fractions						Mean	Standard deviation
	European	Indian	Chinese	Malay	Sēnoi	Negrito		
I	10·1	17·0	13·4	16·0	11·8	*8·5*	12·80	±3·32
II	0·6	*2·3*	0·7	1·1	1·1	1·2	1·17	±0·61
III	6·4	12·0	10·4	*4·2*	8·4	9·5	8·48	±2·82
IV	34·9	33·3	38·4	37·6	*29·3*	29·8	33·88	±3·83
V	30·1	27·1	26·4	28·3	*36·1*	35·6	30·60	±4·26
VI	1·8	1·4	2·5	*6·1*	4·8	4·3	3·48	±1·86
VII	*12·3*	4·2	6·1	4·7	6·5	9·7	7·25	±3·14
VIII	*3·8*	2·7	2·1	2·0	2·0	1·4	2·33	±0·83

For each fraction the figure in italics is the extreme deviate from the mean.

assessing the position of any one of the peaks in the pattern. Thus, for the Malay pattern, where the least quantity of material was applied to the column, the peaks of the fractions after No. 1 are noticeably to the left of those for other ethnic groups.

Table 1 shows the percentage of the total ketosteroid material found in each of the eight fractions for the six ethnic groups. It was shown in the foregoing paper that the mean total daily 17-ketosteroid excretion of the individuals of the various ethnic groups studied decreased in the order: European > Indian > Chinese > Malay > Sēnoi > Negrito. It can be seen in Table 1 that there is none of the eight fractions which decreases in percentage concentration in anything like the same series, except possibly fraction VIII. The small amounts of material involved in this fraction, and the large variations evident in the amounts of the other seven fractions indicate that there may be little or no significance in the finding of this series. As the contents of this fraction have not as yet been identified it is not possible to visualise the possible implications of this finding should it prove to be statistically significant. It is certain that the very small differences in the amounts of 17-ketosteroid material present in the No. VIII fractions of the various ethnic groups cannot account quantitatively for the large differences which were observed in the excretion of total 17-ketosteroid material.

If, for each fraction shown in Table 1, the figure which deviates most from the mean is first excluded, and the mean and standard deviation of the remaining five figures is then calculated, the probability that a normal distribution based on these five figures would include the extreme deviate may be determined. Probability values calculated in this way are less than 1 p.c. in only two cases; Fraction II (the extreme deviate occurs in the Indian ethnic group) and Fraction VIII (extreme deviate in the European ethnic group). These

two are the smallest of the eight fractions, and together represent only 3·5 p.c. of the total eluate 17-ketosteroids for the six ethnic groups. In view of the small size of these two fractions it seems likely that the divergence of the extreme deviate from the mean in each case may be accounted for by the overlap from adjoining fractions.

With the two exceptions which have been discussed, the figures shown in Table 1 give no indication that the various fractions revealed by chromatography on alumina are not present in the extracts of urine from the various ethnic groups in roughly the same proportions. The final significance of the figures in this respect cannot be assessed without further work on the fractions obtained from individual subjects of the various ethnic groups.

The present work may be regarded as extending to a wider section of humanity the implications of the work of Birke and Plantin (1954) who found a similar distribution of 17-ketosteroid material in the fractions obtained from pooled urinary ketosteroids of European men and women of different age groups.

SUMMARY.

Urinary 17-ketosteroid material from Chinese, European, Indian, Malay, Negrito and Sēnoi ethnic groups has been chromatographed on alumina.

The differences between the ethnic groups in the relative amounts of the eight fractions produced by the procedure in no case parallel quantitatively the differences which have been found in total 17-ketosteroid excretion. In general, the relative proportion of the principal fractions are roughly the same in each of the ethnic groups.

Acknowledgment. I wish to thank Mr. Lim Yen Heng for assistance in the design and construction of an automatic fraction collector.

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FURTHER ASPECTS OF THE ANALEPTIC ACTIVITY OF BEMEGRIDE

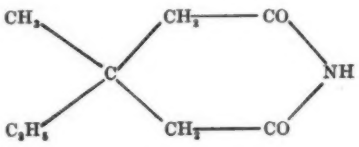
by A. SHULMAN AND GLENDA M. LAYCOCK

(From the Department of Physiology, University of Melbourne).

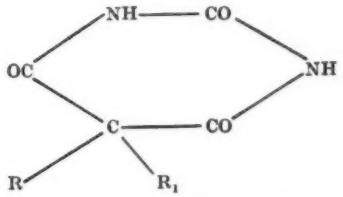
(*Accepted for publication 2nd July, 1957.*)

Some pharmacological properties and clinical uses of Bemegride (I) (β-methyl-β-ethylglutarimide) have been previously considered indicating it to be an effective antagonist to narcosis induced by a wide variety of barbiturates (Shaw, Simon, Cass, Shulman, Anstee and Nelson, 1954; Shulman, Shaw, Cass and Whyte, 1955; Harris, 1955; Holten, 1956; Palmer, 1956, and von Planta and Klinger, 1956). An investigation of its mode of action has indicated some of the structural requirements underlying this activity and has further shown that Bemegride will also relieve the hypnotic effect of substances structurally related to the barbiturates (II), such as the 2:4-diketothiazolidine, 5-phenyl-2:4-diketothiazolidine (III), the monoureides, Bromural (IVA) and Carbromal (IVB) (Shulman, 1956) and the glutarimides, β-methyl-β-(butyl, amyl or hexyl)-glutarimide (V) (Somers, 1956).

This paper extends this work by demonstrating that Bemegride antagonizes the hypnotic activity of another series of substances bearing a structural resemblance to the barbiturate ring system, namely Doriden[1] (VI), (α-ethyl-α-phenylglutarimide) (Gross, Tripod and Meier, 1955); Persedon[2] (VII) 3:3-diethyl-2:4-diketo-1:2:3:4-tetrahydropyridine) (Pellmont, Studer and Jürgens, 1955); Noludar[2] (VIII) (3:3-diethyl-5-methyl-2:4-diketopiperidine) (Pellmont, Studer and Jürgens, 1955), and Dolitrone[3] (IX) 5-ethyl-6-phenyl-2:4-diketo-metathiazane) (Lundy, 1955).

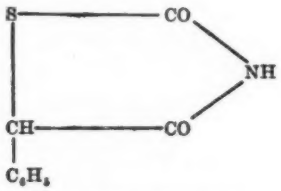


I. Bemegride
(β-methyl-β-ethylglutarimide)

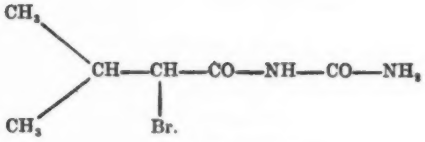


II. An α:α-(5:5)-disubstituted barbiturate

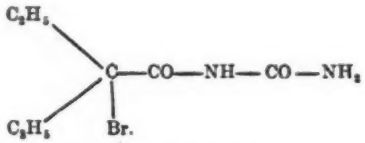
[1] C.I.B.A. (Basel).
[2] F. Hoffman-La Roche & Co. A. G. (Basel).
[3] William S. Merrell & Co. (U.S.A.).



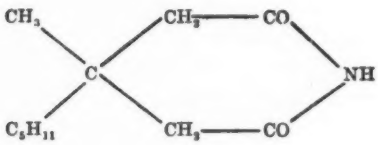
III. 5-phenyl-2:4-diketothiazolidine



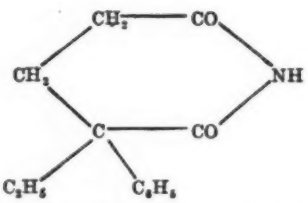
IVA. Bromural
(α-bromo-β-methyl-butyrylurea)



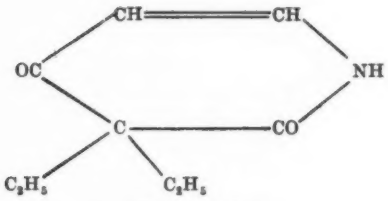
IVB. Carbromal
(α-bromo-α-ethyl-butyrylurea)



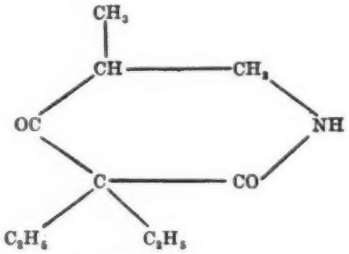
V. β-methyl-β-amylglutarimide



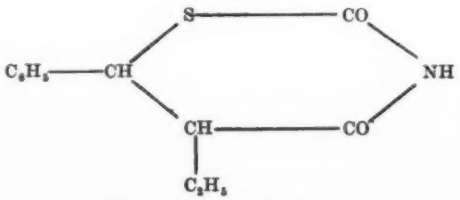
VI. Doriden (α-ethyl-α-phenylglutarimide)



VII. Persedon
(3:3-diethyl-2:4-diketo-1:2:3:4-tetrahydropyridine)



VIII. Noludar
(3:3-diethyl-5-methyl-2:4-diketopiperidine)



IX. Dolitrone (5-ethyl-6-phenyl-2:4-diketometathiazane)

Methods.

Coloured mice of mixed strain and sex weighing between 25 and 30 gm. were divided by random selection into test groups. The analeptic potency of Bemegride was estimated from its ability to reduce the sleeping time of mice given a prior injection of a selected hypnotic (Dolitrone, Doriden, Noludar and Persedon). The dose of hypnotic was chosen so as to give a mean sleeping time for the control group of 60-80 minutes. The sleeping time was considered as the interval elapsing between the loss and the maintained recovery of the righting reflex. All drugs were administered intraperitoneally either in saline solution (Bemegride, Dolitrone, Noludar and Persedon) or when necessary, in a 1 p.c. tragacanth suspension (Doriden). Bemegride or saline was administered at 15 minute intervals.

A "t" test was carried out in all cases where there appeared to be a significant decrease in the sleeping time.

TABLE 1.

	Persedon 250 mg./kg.		Noludar 185 mg./kg.	
Treatment: Saline	+	−	+	−
Treatment: Bemegride	−	20 mg./kg.	−	20 mg./kg.
Number of animals taken	20	20	20	20
Number of survivors used in calculations	20	20	20	20
Mean sleeping time (minutes)	64·75	31·85	79·05	24·05
Standard Error (S.E.)	4·49		7·01	
"t" test versus saline	7·33***		7·87***	

*** = highly significant ($P < 0{\cdot}001$)

TABLE 2.

	Doriden 125 mg./kg.		Dolitrone 225 mg./kg.	
Treatment: Saline	+	−	+	−
Treatment: Bemegride	−	20 mg./kg.	−	20 mg./kg.
Number of animals taken	20	20	20	20
Number of survivors used in calculations	20	20	20	19
Mean sleeping time (minutes)	73·55	12·05	60·95	15·37
Standard Error (S.E.)	7·05		4·22	
"t" test versus saline	8·73***		10·80***	

*** = highly significant ($P < 0{\cdot}001$)

RESULTS.

The effect of Bemegride (20 mg./kg.) in relieving narcosis induced in mice by Noludar (185 mg./kg.) and Persedon (250 mg./kg.) is seen in Table 1. Its ability to reverse the hypnotic activity of Dolitrone (200 mg./kg.) and Doriden (125 mg./kg.) is seen in Table 2.

DISCUSSION.

This and previous work (Shaw *et al.*, 1954; Shulman, 1956; Somers, 1956) has indicated the ability of Bemegride to relieve hypnosis induced in animals by barbiturates (pentobarbitone sodium; thiopentone sodium) and such structurally related cyclic hypnotics as the glutarimides (Doriden and β-methyl-β-(butyl, amyl or hexyl)-glutarimide), the diketopiperidine, Noludar, the diketotetrahydropyridine, Persedon, the diketothiazane, Dolitrone and the diketothiazolidine, 5-phenyl-2:4-diketothiazolidine. A similar antagonism by Bemegride to the hypnotic action of the open chain hypnotics, such as the monoureides, Bromural and Carbromal, has also been demonstrated.

These observations on animals parallel those resulting from the clinical use of Bemegride which similarly reverses the syndrome of acute narcotic coma due to deliberate or therapeutic overdosage with many of these substances (barbiturates, glutarimides, monoureides, 2:4-diketopiperidines and 2:4-diketotetrahydropyridines) (Harris, 1955; Shulman *et al.*, 1955; Holten, 1956 and von Planta and Klinger, 1956). Bemegride also quickly relieves the symptoms, signs and E.E.G. pattern of chronic barbiturate intoxication (Palmer, 1956).

SUMMARY.

Bemegride, which antagonizes hypnosis induced in mice by barbiturates (pentobarbitone sodium and thiopentone sodium), the monoureides (Bromural and Carbromal), the diketothiazolidine, 5-phenyl-2:4-diketothiazolidine and the glutarimides (β-methyl-β-(butyl, amyl or hexyl)-glutarimide), has been shown also to reverse the hypnotic activity of the structurally related glutarimide, Doriden, the diketopiperidine, Noludar, the diketotetrahydropyridine, Persedon and the diketothiazane, Dolitrone.

Acknowledgments. We wish to thank C.I.B.A. (Basel), the William S. Merrell Co. (U.S.A.) and F. Hoffman-La Roche & Co. (Basel) for their generous gifts of drugs used in this investigation.

We are indebted to Mr. J. Robilliard for technical assistance.

We are most grateful to Dr. K. H. Pausacker for his helpful criticism and to Professor R. D. Wright for his advice and guidance during this investigation.

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FLAVOKINASE OF LACTOBACILLUS ARABINOSUS 17.5

by A. M. SNOSWELL[1]

(From the Department of Biochemistry, University of Adelaide).

(*Accepted for publication 31st May, 1957.*)

The synthesis of flavin mononucleotide (FMN)[2] by phosphorylation of riboflavin in intestinal mucosa (Pulver and Verzar, 1939) and also the synthesis of flavin adenine dinucleotide (FAD) from riboflavin in human blood cells (Klein and Kohn, 1940) have been reported. Siliprandi *et al.* (1955) have demonstrated FAD synthesis from riboflavin and ATP in cells isolated from rat liver homogenates. Schneider and Hogeboom (1956) obtained synthesis of FAD from FMN and ATP by using a soluble fraction isolated from rat liver.

However, biosynthesis of flavin nucleotides has been studied in detail only in yeast. Kearney and Englard (1951) have purified from brewer's yeast, an enzyme (flavokinase), which catalyses the reaction.

$$\text{riboflavin} + \text{ATP} \rightarrow \underset{(\text{FMN})}{\text{riboflavin 5' phosphate}} + \text{ADP} \quad .. \quad .. \quad (1)$$

Previously, Schrecker and Kornberg (1950) had isolated, from brewer's yeast, an enzyme, adenyl transferase, which catalyses the reaction.

$$\text{FMN} + \text{ATP} \rightarrow \text{FAD} + \text{PP} \quad .. \quad .. \quad .. \quad .. \quad .. \quad (2)$$

It has been found (Hunt, 1956; c.f. text) that cell-free extracts of *Lactobacillus arabinosus* 17·5 contain flavin in the form of FMN and FAD rather than free riboflavin.

The synthesis of flavin nucleotides from riboflavin in cell-free extracts was therefore investigated. On fractionation of cell-free extracts, it was found that adenyl transferase activity (reaction 2) was associated with a yellow flavoprotein fraction. This made difficult the measurement of small increments in FAD against a high flavin blank. Fractions with flavokinase activity, on the other hand, were easily separated from this fraction and were thus more suitable for study. The present paper describes the purification and properties of a flavokinase containing fraction from cell-free extracts.

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[1] This work was undertaken during the tenure of a C.S.I.R.O. Australian Studentship.

[2] The following abbreviations have been used in this paper:
FMN: flavin mononucleotide (riboflavin 5' phosphate); FAD: flavin adenine dinucleotide; AMP: adenosine monophosphate; ADP: adenosine diphosphate; ATP: adenosine triphosphate; CTP: cytosine triphosphate; GTP: guanosine triphosphate; ITP: inosine triphosphate; UTP: uridine triphosphate; PP: pyrophosphate; Tris (hydroxy methyl): amino methane; EDTA: ethylene-diamine-tetra-acetic acid.

Methods and Materials.

Cells of *Lactobacillus arabinosus* 17·5 were grown in 8 litre batches at 30° C. for 16 hours in a Pronutrin-glucose medium described by Nossal (1952) and supplemented with 80 ml./l. yeast extract (prepared by boiling Effront baker's yeast with an equal volume of distilled water and filtering). The cells were harvested in a Sharples centrifuge, washed twice with distilled water and suspended in 0·1M Tris buffer (pH 7·0) to give a concentration of approximately 45 mg./ml. dry wt. of cells. Approximately 0·5 gm. dry wt. cells were produced per litre of culture media.

Preparation of the extract: Cell-free extracts were prepared by 90 sec. disintegration with glass beads at 0-2° C. in an ultra-rapid shaker (Nossal, 1953) oscillating at 3,000 cycles per min. Routinely, the capsules were charged with 15 ml. of cell suspension (containing approx. 45 mg. cells dry wt./ml.) in 0·1M Tris buffer (pH 7·0) and 10 gm. Ballotini No. 14 glass beads. The glass beads were allowed to settle in the cold and the supernatant centrifuged at 20,000 *g* for 20 min. at 0° C. in an angle head Sorvall refrigerated centrifuge. The cell-free supernatant was a pale straw colour and contained approx. 12 mg. protein/ml.

Reagents: All nucleotides were purchased from the Sigma Chemical Co. ATP (disodium trihydrate) and ADP (disodium salt) were chromatographically pure and were not purified further. CTP, GTP, UTP in the sodium form and ITP (barium salt) were chromatographically free from ATP. Riboflavin and FMN (mono-sodium salt) were used without further purification.

Estimations: Riboflavin, FMN and FAD were estimated by the method of Burch, Bessey and Lowry (1948) using a Farrand-Fluorometer. Estimates were accurate to ± 5 p.c. within the range 0·1 to 1·0 millimicro mole./ml. Protein was estimated by the biuret method (Robinson and Hodgen, 1940).

Enzyme assay: All assays and subsequent analyses were carried out in a darkened room to prevent photo-decomposition of the riboflavin compounds.

The enzyme was routinely assayed in a system containing 2 μ mole. ATP, 1·5 μ mole. $MgCl_2$, 75 μ mole. Tris buffer (pH 7·0) and 10 mμ mole. riboflavin in a volume of 1 ml., to which was added an aliquot of enzyme and distilled water to give a total volume of 2 ml. The mixture was incubated in a water bath at 30 ° C. for 1 hour. The reaction was stopped by transferring a 0·5 ml. aliquot to 3·5 ml. ice-cold 3 p.c. trichloroacetic acid. After standing for 15 min. at 4° C. the protein precipitate was removed by centrifugation at 1000 *g*. for 10 mins. Aliquots were taken for flavin assays. All assays were corrected for reagent and enzyme blanks.

A unit of activity was defined as that amount causing the synthesis of 1 mμ mole. FMN in 1 hour under the conditions of assay, and specific activity as units/mg. protein.

Chromatography of reaction products: In order to study the small changes in adenine nucleotides involved in the flavokinase reaction, it was necessary to chromatograph much larger aliquots (0·5 ml.) than normal. Therefore, the salt concentration had to be kept minimal, and trichloroacetic acid was omitted. After incubation of 1·0 ml. of the concentrated enzyme fraction (see under purification) with 200 mμ mole. ATP, 30 μ mole. Tris buffer (pH 7·0) 1·5 μ mole. $MgCl_2$, and 100 mμ mole. riboflavin in a total volume of 2 ml., the reaction was stopped by immersing the tubes in boiling water for 30 secs. The denatured protein was removed by centrifugation and the clear supernatant was examined chromatographically for adenine nucleotides by the method of Krebs and Hems (1953). 0·5 ml. aliquots of supernatant were applied, 36 cm. from one end, to Whatman No. 1 filter paper (5·5 × 70 cm.), previously washed with 0·1M EDTA (pH 8·0). An automatic continuous spotting device, which consisted of an electric clock motor geared down to 1 rev./2 min. driving an "Agla" micrometer syringe (Burroughs Wellcome Co.) was used to apply these large aliquots. The papers were first run in n-butanol, acetic acid, water solvent

(5: 1: 4) for 3 hours (in the opposite direction to that for development of adenine nucleotides) in order to remove flavins. Adenine nucleotides remain on the base line in this solvent.

The papers were dried for 30 mins. in a stream of warm air and then cut off 3 cm. from the application line. The papers were developed for 16 hours by ascending chromatography in a solvent composed of redistilled iso-butyric acid, N ammonia and 0·1M EDTA (pH 7·0) in the ratio 20: 12: 0·3. The spots were detected by spraying with 0·1 p.c. ferric chloride in 80 p.c. ethanol followed by 1 p.c. salicyl sulphonic acid in 80 p.c. ethanol as described by Wade and Morgan (1953). Fresh ferric chloride solution was prepared each time, before use.

Using this technique, it was possible to detect down to 25 mμ mole. of ADP and 8 mμ mole. of ATP.

Flavin compounds were chromatographed in the dark to prevent photo-decomposition. 20 μl. spots of reaction mixture (see above) were applied 3 cm. from one end of 5·5 × 40 cm. Whatman No. 1 papers.

The ascending chromatograms were developed for 16 hrs. in the n-butanol-glacial acetic-water (5: 1: 4) solvent described by Crammer (1948). After drying, flavin spots were detected by their fluorescence in ultra-violet light.

Determination of Km values: In experiments involving the determination of Michaelis constants (Km values) by the Lineweaver-Burk plot method, a 10 min. incubation period was used instead of the usual 1-hour period. The shorter 10 min. period was desirable as substrate concentrations were then nearer their initial values. Alberty (1956) previously suggested such a precaution was necessary for the determination of Km values by this method.

RESULTS.

Identification of Flavins present in Cell-free Extracts.

Identification of flavins in cell-free extracts showed that the flavin present occurred mainly as FAD with some FMN and little or no free riboflavin (Table 1). The ratio of FAD to FMN was approximately 7 : 1, whereas Haley and Lambooy (1954) found the ratio to be 16 : 1 (with no free riboflavin) in cells of *Lb. casei*.

TABLE 1.

The distribution of flavins in cell-free extracts.
Data in mμ mole./ml.

No. of assays	riboflavin		FMN		FAD		Protein mg./ml.
	Extremes	Mean	Extremes	Mean	Extremes	Mean	Mean
4	0—0·9	0·4	0·9—1·5	1·2	3·3—13·3	8·3	11·3

All figures corrected for blanks.

Flavin Nucleotides synthesised by crude Cell-free Extracts.

In crude, cell-free extracts, synthesis of FMN from riboflavin was generally 50 p.c. greater than the synthesis of FAD from FMN (Table 2). In several batches of extract, FAD synthesis could not be detected at all, but could be

TABLE 2.

Biosynthesis of flavin nucleotides by cell-free extracts.

FMN synthesized mμ mole./ml.		FAD synthesized mμ mole./ml.	
Complete assay system	2·2	Complete assay system	1·6
Minus ATP	0	Minus ATP	0
Minus riboflavin	0	Minus FMN	0
Minus $MgCl_2$	2·1	Minus $MgCl_2$	1·6

Reaction mixture contained 2 μ mole. ATP, 1·5 μ mole. $MgCl_2$, 75 μ mole. Tris buffer (pH 7·0) and 10 mμ mole. riboflavin or FMN. Incubated at 30°C.

demonstrated after further fractionation. This could have been due to the subsequent removal of inhibitory agents or enzymes such as nucleotide pyrophosphatase; the latter would catalyse the breakdown of FAD to FMN and AMP, so that no net synthesis of FAD would be observed.

Enzyme Fractionation and Purification.

All procedures described below were carried out in a cold room at 0-2° C.

The cell-free extract was treated with ice-cold 3 p.c. protamine sulphate (pH 7·0) to remove nucleic acids (0·1 mg. protamine sulphate to 1 mg. protein was required to reduce the E_{280}/E_{260} ratio to less than 1·0). The protamine treated extract was centrifuged at 1,500 g. for 10 min. in a refrigerated angle-head Sorvall centrifuge. The supernatant was poured off and brought to 0·6 saturation with solid ammonium sulphate (determined from the chart given by Green and Hughes, 1955, and corrected to 0° C.). The pH dropped from 7·0 to 6·7 during this addition. After standing overnight at 0-2° C., the precipitated protein was collected by centrifugation at 20,000 g., and redissolved in ice-cold 0·1M Na/K phosphate buffer (pH 7·0) to give a final concentration of approx. 10 mg. protein/ml. This solution was dialysed for 4 hours against glass-distilled water and then brought to pH 5·5 with 0·2M H_3PO_4 and calcium phosphate gel added with constant stirring (1·5 mg. gel/mg. protein). The gel was removed by centrifugation at 0° C., washed with one volume 0·01M acetate buffer (pH 5·5) and the washings added to the gel supernatant.

This treatment resulted in the absorption of 80 p.c. of the total protein onto the gel, including all the adenyl transferase activity. The combined supernatant and washings contained the flavokinase: a 40-fold increase in purity was achieved (Table 3).

For experiments involving the identification of reaction products a more concentrated solution of the enzyme was required. For this purpose, the supernatant obtained after calcium phosphate gel treatment was dialysed overnight against glass-distilled water, lyophilized in a freeze-drying apparatus and redissolved in 1/10 the original volume of distilled water.

TABLE 3.

Fractionation of flavokinase.

Fraction	Volume (ml.)	Protein mg./ml.	Total protein	Activity units/ml.	Total units	Specific activity	Increase in activity	Yield
Crude cell-free extracts	105	12·3	1,290	1·60	168	0·13		
0—0·6 ammon. SO_4 fraction after protamine SO_4 treatment	17	12·0	203	12·7	193	1·06	8 fold	115%
Ca phosphate gel supernatant	27	0·8	22	4·0	109	4·95	38 fold	65%

Standard assay conditions as in Table 2.

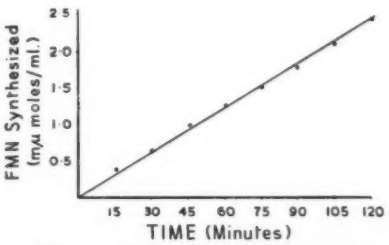

Fig. 1. The rate of FMN synthesis. Reaction mixture contained 2 μ mole. ATP, 1·5 μ mole. $MgCl_2$, 75 μ mole. Tris buffer (pH 7·0) and 10 mμ mole. riboflavin. Incubated at 30° C. with 0·4 mg. enzyme in a total volume of 2 ml.

Properties of the Flavokinase.

Stability: Final fractions retained 90 p.c. of their enzymic activity after storage at — 15° C. for 2 months. Lyophilization also proved an effective method of storing the final fraction.

Kinetics: Enzyme activity followed a zero-order reaction for 2 hours (Fig. 1) and was directly proportional to enzyme concentration in the range 0·08 to 0·8 mg. protein (Fig. 2). Optimal enzyme activity was observed over the pH range 6·5 to 7·0 (Fig. 3).

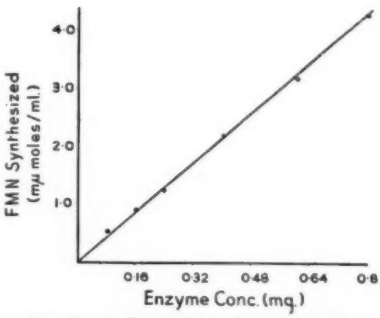

Fig. 2. Relationship of FMN synthesis and enzyme concentration. Standard assay conditions as in Fig. 1. Incubated at 30° C. for 1 hour.

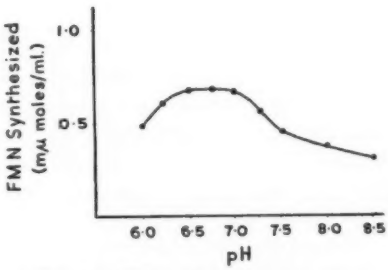

Fig. 3. Effect of pH on enzyme activity. Standard assay conditions as in Fig. 1, except Na/K phosphate buffer used in the range pH 6·0-7·5.

Substrate Requirements: The enzyme was half-saturated with riboflavin at a concentration of $3{\cdot}0 \times 10^{-6}$M (Fig. 4b), compared with the yeast flavokinase at $1{\cdot}0 \times 10^{-5}$M. Phosphorylation of riboflavin in this system showed an abso-

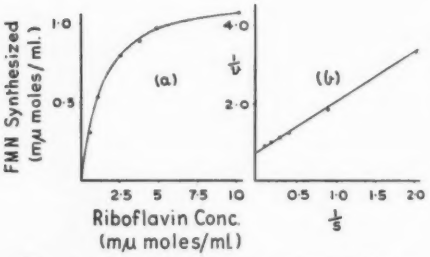

Fig. 4. Effect of riboflavin concentration on enzyme activity.
(a) FMN synthesized against riboflavin conc. Standard assay conditions as in Fig. 1. Incubation period 10 mins. Enzyme 0·45 mg./tube.
(b) Lineweaver-Burk plot. Ordinate, 1/v where v = mμ mole. FMN synthesized/ml. reaction mixture. Enzyme and assay conditions as in (a).

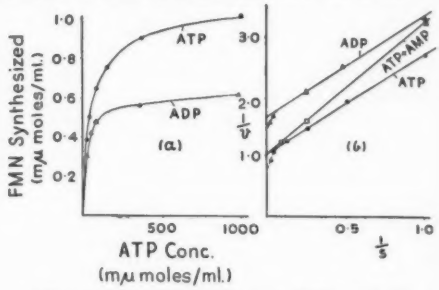

Fig. 5. Effect of ADP and ATP on enzyme activity.
(a) FMN synthesized against ATP and ADP conc. Standard assay conditions as in Fig. 1. 10 min. incubation.
(b) Lineweaver-Burk plot. Ordinate, 1/v where v = mμ mole. FMN synthesized/ml. reaction mixture. Abscissa, 1/s where s = conc. of ADP or ATP in mμ mole./ml. reaction mixture. 2 μ mole. AMP used for competitive inhibition. Enzyme and assay conditions as in (a).

lute requirement for a high-energy phosphate donor, which could be met by either ADP or ATP. The Km for ADP was $1{\cdot}2 \times 10^{-5}$M while that for ATP was $1{\cdot}6 \times 10^{-5}$M (Fig. 5b). The Lineweaver-Burk plots for ATP and ADP (Fig. 5b) show a "tailing off" which is characteristic of an enzyme requiring two substrates. AMP acts as a competitive inhibitor of ATP (Fig. 5b). ADP was not as effective as ATP as a phosphate donor (Fig. 5a). The observed activity of ADP in this system could have been due to myokinase which catalyses the reaction 2 ADP $\rightleftharpoons$ ATP + AMP, and would thus provide ATP. However, no myokinase activity could be detected using chromatographic techniques, as indicated by the following experiment. After incubation of the enzyme with 2 μ mole. ADP, 1·5 μ mole. $MgCl_2$, and 75 μ mole. Tris buffer (pH 7·0) for 1 hour, the reaction was stopped with ice-cold 5 p.c. trichloroacetic acid. The reaction mixture was then centrifuged and aliquots of the supernatant were examined chromatographically as described under "Methods". Only ADP was found to be present; the absence of AMP and ATP excluded myokinase activity. Yeast flavokinase can also utilize ADP as a phosphate donor (Kearney and Englard, 1951).

Other Phosphate Donors: UTP at the same concentration as ATP was found to be approximately 60 p.c. as effective as a phosphate donor, but CTP, GTP and ITP were inactive.

Metal Ion Requirements: Flavokinase activity in the original cell-free extract, showed no stimulation on the addition of metal ion. However, the gel supernatant fraction was stimulated by added metal ions, Mg^{++} being most active. After 16 hours' dialysis against 0·1M EDTA (pH 6·0) an absolute requirement for divalent ion was observed. Mg^{++} was most effective, Mn^{++} stimulated slightly and Ca^{++}, Co^{++}, Fe^{++}, Mo^{++}, Ni^{++} and Zn^{++} were inactive. Stimulation by Mg^{++} was optimal at $3{\cdot}0 \times 10^{-3}$M. Higher concentrations inhibited enzyme activity (Fig. 6).

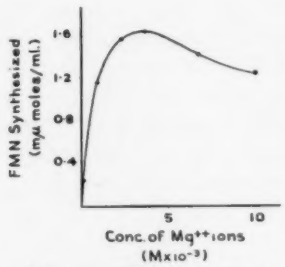

Fig. 6. Effect of Mg^{++} concentration. Standard assay conditions as in Fig. 1. 0·45 mg. enzyme (dialysed overnight against 0·1M EDTA).

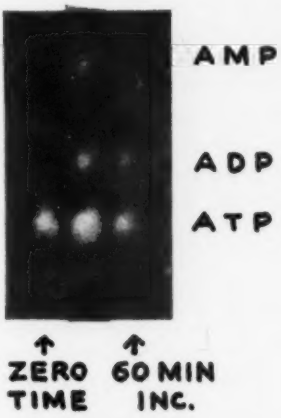

Fig. 7. Chromatogram of reaction product. Reaction mixture contained 200 mμ mole. ATP, 1·5 μ mole. $MgCl_2$, 3 μ mole. Tris buffer (pH 7·0) and 100 mμ mole. riboflavin. Incubated at 30° C. for 60 mins. with 10·5 mg. enzyme. Reaction stopped by immersing for 30 secs. in boiling water, 0·5 ml. supernatant chromatographed.

Identification of Reaction Products: The amount of FMN formed during the incubation of the enzyme under standard conditions equalled the amount of riboflavin used (Table 4). FMN was also identified chromatographically as a reaction product. Using ATP as a phosphate donor, ADP was identified chromatographically as the other reaction product (Fig. 7). In order to demonstrate a disappearance of ATP and a parallel formation of ADP the assay mixture was altered: 200 mμ mole. ATP and 100 mμ mole. riboflavin were incubated with 10·5 mg. concentrated enzyme fraction which converted approxi-

TABLE 4.

Stoichiometry of the flavokinase reaction.

Riboflavin used m μ mole./assay	FMN produced m μ mole./assay
1·9	1·8
2·0	1·9
2·1	2·1
1·9	2·0
2·1	2·2
Mean 2·0	Mean 2·0

Standard assay conditions as in Table 2
0·4 mg. enzyme/assay.

mately 80 mμ mole. riboflavin to FMN. Of the total 2·0 ml. reaction mixture, 0·5 ml. was applied to the chromatograms (see Methods). The appearance of ADP after incubation with the enzyme fraction could have been due to the presence of ATP 'ase activity. However, after incubation of the enzyme with ATP in the absence of riboflavin, only ATP could be detected chromatographically. The reaction catalysed by the flavokinase of *Lb. arabinosus* therefore may be formulated in exactly the same way as yeast flavokinase, viz.:

$$\text{riboflavin} + \text{ATP} \rightarrow \text{FMN} + \text{ADP}.$$

DISCUSSION.

Lb. arabinosus requires riboflavin for optimal growth (Snell, 1951), while another member of this genus, *Lb. casei,* has an absolute requirement for riboflavin and is used in the microbiological assay of this vitamin (Snell, 1951).

The current experiments show that *Lb. arabinosus* like *Lb. casei* (Haley and Lambooy, 1954) contains riboflavin in the nucleotide form as FMN and FAD. It can be calculated that *Lb. arabinosus* cells contain 0·018 and 0·003 μ mole. FAD and FMN respectively/gm. dry weight. The value for FAD compares well with that recently published by Deluca, Weber and Kaplan (1956).

In all cases, where riboflavin is necessary as a growth factor the vitamin in the free form suffices, indicating that the nucleotide may be synthesized within the cell.

In spite of the universal occurrence and wide importance of flavoproteins, the only previous studies concerning the biosynthesis of FAD and FMN have involved brewer's yeast; Kearney and Englard (1951) have purified yeast flavokinase some 500-fold. The *Lb. arabinosus* enzyme, although only purified 40-fold, is similar in many respects, e.g. results described here show similar dissociation constants, metal ion requirements, high energy phosphate donors, etc. On a dry weight basis, brewer's yeast appears to contain twice as much flavokinase as *Lb. arabinosus.* The adenyl transferase, discovered in brewer's yeast by Schrecker and Kornberg (1950) has not been studied in detail in *Lb.*

arabinosus, but synthesis of FAD from FMN and ATP has been observed in cell-free extracts (Table 2).

Both brewer's yeast and *Lb. arabinosus* are essentially anaerobic organisms. Both not only contain riboflavin in the nucleotide form, but actually possess the enzymes necessary for flavin nucleotide synthesis. Of the micro-organisms examined for flavin content, Deluca *et al.* (1956) found anaerobic organisms to have the highest flavin content. Yet, as Mahler (1956) points out, little is known of the rôle of flavoproteins in anaerobes. Although Barron (1951) has suggested the latter may satisfy their energy-requirements *via* flavoprotein-linked oxidation-reduction reactions, experimental evidence is scant; Shug *et al.* (1954) and Whiteley and Ordal (1955) have purified hydrogenase from *C. pasteurianum* and *M. lactilyticus* respectively, both enzymes apparently being molybdo-flavoproteins. The latter could be coupled to xanthin oxidase (another molybdo-flavoprotein) *via* one-electron dyes (e.g. benzyl viologen) or a naturally occurring, unknown, iron-containing carrier.

However, such subsidiary functions do not explain the universal distribution nor the high content of flavin nucleotides in anaerobic cells. Investigations are therefore being undertaken to ascertain the rôle of flavin nucleotides in the metabolism of *Lb. arabinosus*.

Note added in proof: As yeast hexokinase specifically catalyses the reaction ATP + glucose → ADP + glucose -6-PO_4, it can be used to remove even traces of ATP. Addition of 0·5 unit hexokinase and 100 μ mole glucose completely abolished phosphorylation of riboflavin with ATP, ADP and UTP as phosphate donors. This proves that ADP and UTP cannot act as direct donors. Thus the samples of ADP and UTP used either contained small amounts of ATP or myokinase was present at a level not detectable by the chromatographic methods used. ATP is therefore the only true phosphate donor in the flavokinase reaction.

SUMMARY.

Cell-free extracts of *Lactobacillus arabinosus* 17·5, prepared by mechanical disintegration of cells with glass beads, contained flavin in the form of FMN and FAD rather than free riboflavin. The ratio of FMN to FAD was 1 : 7. Synthesis of both FMN and FAD in cell-free extracts has been demonstrated.

Approximately 40-fold purification of a flavokinase from these extracts has been described. The flavokinase catalyses the reaction:

riboflavin + ATP → FMN + ADP.

In addition to ATP, both ADP and UTP may act as phosphate donors; CTP, GTP and ITP were inactive while AMP exhibited competitive inhibition. Dissociation constants for riboflavin, ATP and ADP have been determined.

A divalent metal ion was required for optimal activity, Mg^{++} being most effective.

Both FMN and ADP were identified chromatographically as reaction products when ATP was the phosphate donor.

The similarity of this enzyme to the yeast flavokinase has been discussed.

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ELECTRON MICROSCOPY OF THE MARSUPIAL RENAL GLOMERULUS

by R. K. F. PAK POY

(From the Department of Pathology, University of Adelaide).

(*Accepted for publication 4th July, 1957.*)

The structure of the glomerulus in higher mammals has been described by many workers, using light microscopy (Bowman, 1842; Von Mollendorf, 1928; Vimtrup, 1928; McGregor, 1929; McManus, 1948; Bell, 1950; Allen, 1951), and with the electron microscope (Pease and Baker, 1950; Hall, 1953a; Rhinehart, 1953; Reid, 1954; Pease, 1955). Work has also begun in this laboratory with the electron microscope on the comparative morphology of the glomerulus and the glomerulus of the bird has been described by Pak Poy and Robertson (1957). The present paper reports a study of the glomerulus of the opossum (*Trichosurus vulpecula*).

Materials and Methods.

Animals of unknown age were used. Anaethesia was induced with ether and continued with intra-peritoneal nembutal, supplemented with inhaled ether. The kidney was exposed, the capsule removed, the surface scarified and 1 p.c. osmium tetroxide buffered with acetate-veronal at pH 7·4 (Palade, 1952), dropped on for five minutes. A piece of cortex was removed, sliced into 1 mm. cubes and these were left in the above fixative for two hours at 4° C. After fixation the tissue blocks were washed, dehydrated, infiltrated with pure methacrylate and embedded in 20 p.c. methyl methacrylate in butyl methacrylate. Sections were cut with glass knives in a thermal-expanding microtome, mounted on parlodian-coated grids and examined in a Metropolitan-Vickers EM4 electron microscope at 50 k.v. using a 100 μ objective aperture.

Thicker sections (0·5 μ) were mounted on glass slides, dipped in toluene to remove the methacrylate and stained by Jones's silver method (Jones, 1951) and finally viewed by phase contrast.

RESULTS.

Glomerular loop.

The glomerular capillary loops are as numerous and as tortuous as in higher mammals and are characteristically lined by a thin layer of endothelial cytoplasm which contains dense granules and is bound by cytoplasmic membrane (Fig. 1). The thickness of this sheet is variable measuring from 300Å to 3000Å but there are also scattered small ridges, which may contain mitochondria, projecting into the lumen of the capillary loop. Irregularly scattered throughout

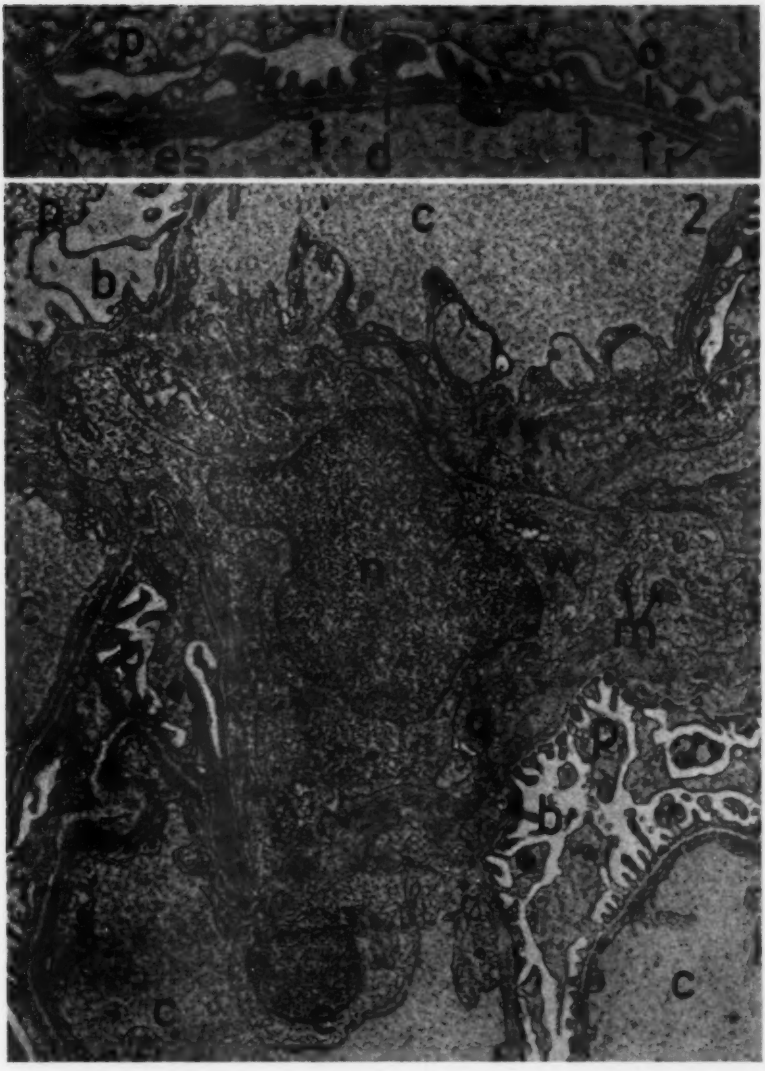
this thin sheet of cytoplasm are numerous pores measuring 250Å to 800Å in diameter and at their margins the inner and outer endothelial cytoplasmic membranes become continuous. Oblique section through an area with pores shows the "chicken wiring" first described and called the lamina fenestrata by Hall (1953a) and later by Pease (1955) and Yamada (1955a). The main cell body with nucleus is usually situated against the intercapillary tissue from which it is separated by intercellular substance and its cytoplasm contains mitochondria, dense granules, endoplasmic reticulum and vacuoles some of which are extremely large. Similar vacuoles have been reported by Reid (1954) and Bergstrand (1957); they are probably due to poor fixation. These very large "balloons" do not resemble the network of vacuoles reported in the endothelium of the avian renal glomerulus by Pak Poy and Robertson (1957). The nuclei are usually ovoid and frequently do not contain nucleoli.

Basement membrane of glomerular loop.

In general, the basement membrane is composed of the three layers described by Rhodin (1955) in unstated animal, Pease (1955) in rats, Yamada (1955a) in mice and Bergstrand (1957) in rabbits, dogs, mice and rats. There is an outer less dense layer, a middle dense layer and an inner less dense layer (Fig. 1). However, the middle dense layer sometimes divides into two before it enters the intercellular space of the intercapillary tissue and occasionally it is composed of three to four thin laminae (Fig. 4); or it may form a syncytium in which the spaces are filled with less dense material similar to that of the less dense layers of the basement membrane (Fig. 5). The basement membrane is continuous with the intercellular substance of the intercapillary tissue, separating intercapillary cells from the epithelial pedicels lying in Bowman's space, until it becomes continuous with the basement membrane of the next capillary loop. In the silver-stained sections (Fig. 9) the basement membrane is shown very clearly and it passes around from capillary loop to capillary loop as it invests the glomerulus as a whole.

Epithelial cells.

These are large cells with large nuclei and abundant cytoplasm, and lie free in Bowman's space. Large and small pedicels (Hall, 1953a) arise from the main mass of cytoplasm and project on to the outer less dense layer of the capillary loop basement membrane and the surface of the intercapillary tissue exposed to Bowman's space. A thin, short membrane which Yamada (1955a) in mice called the filtration slit membrane can also be seen in the opossum, joining adjacent pedicels in some regions and being absent in other regions. It is usually placed further out in Bowman's space than the pedicel-basement membrane junction (Fig. 1). Where this membrane is absent the capillary loop basement membrane is exposed to Bowman's space. The pedicels may interdigitate to form a complex pattern which is particularly well seen in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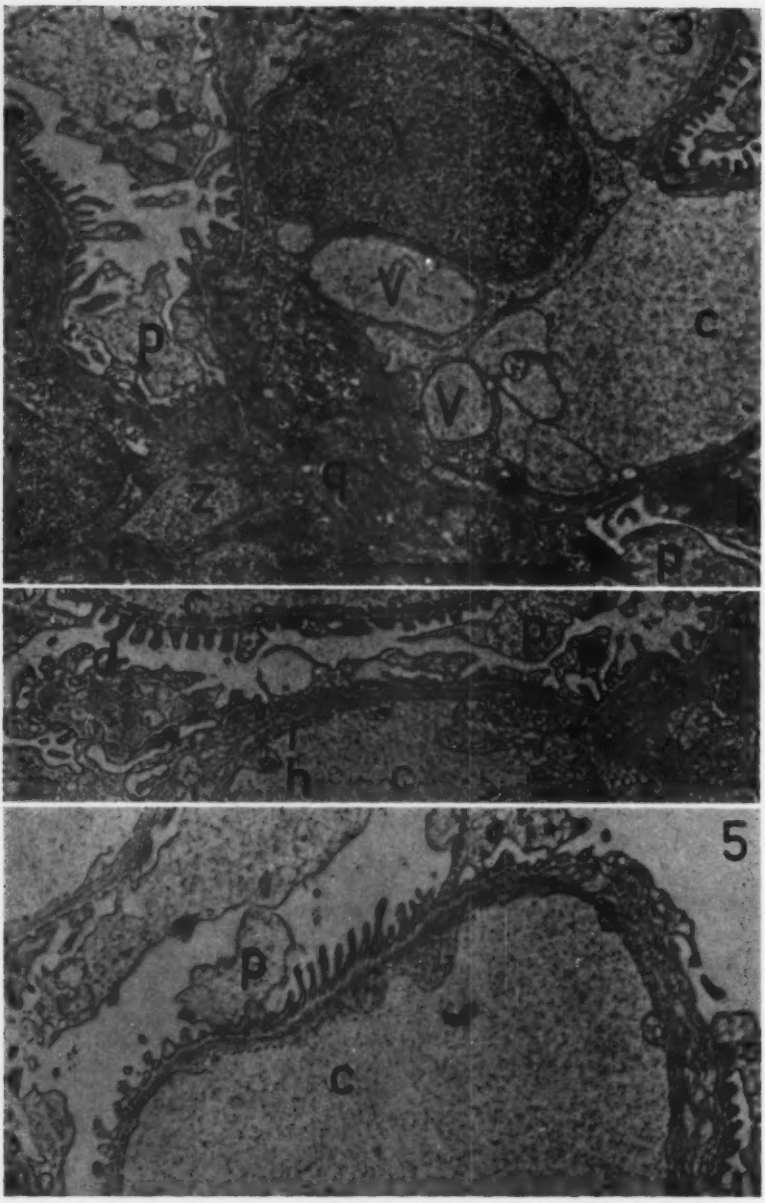
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Fig. 1. The micrograph shows a perpendicular section through the wall of a capillary loop of the glomerulus with pedicels (p) resting on the outer less dense layer of the basement membrane (o). The thin endothelial sheet (es) with projections and pores (indicated by arrows) lines the capillary lumen. The middle dense layer of the basement membrane is shown at (d) and divides into two at the left side, while the inner less dense layer is at (i). The filtration slit membrane is clearly shown at (t). × 18,000.

Fig. 2. The micrograph shows an intercapillary cell (w) with nucleus (n) and mitochondria at (m). The dense homogeneous intercellular substance is at (q). The lumen of four capillary loops are at (c) while Bowman's space is shown at (b). An obvious endothelial cell (e) with the cytoplasm containing mitochondria is at the lower end of the micrograph. Pedicels (p) are present in Bowman's space and project onto the intercapillary tissue. × 12,000.

Fig. 3. The micrograph shows a nucleus (y) of an endothelial cell with large vacuoles in the cytoplasm at (v). Pedicels are shown at (p) and intercellular material of the intercapillary tissue at (q). A projection of cytoplasm in the intercapillary tissue is shown at (z) and a capillary lumen at (c). × 12,000.

Fig. 4. The micrograph shows two adjacent capillary walls of which the higher one shows the lamina densa (d) as a single layer, while the lower one shows the lamina densa in threads at (h). Pedicels are shown at (p) and capillary lumens at (c). × 15,000.

Fig. 5. The micrograph shows the dense middle layer of the capillary loop basement membrane as a single layer on the left side. The middle of the micrograph shows tangential sectioning of the capillary wall and the right side shows the dense layer breaking up into a syncytium. Pedicels are shown at (p) and capillary lumen at (c).

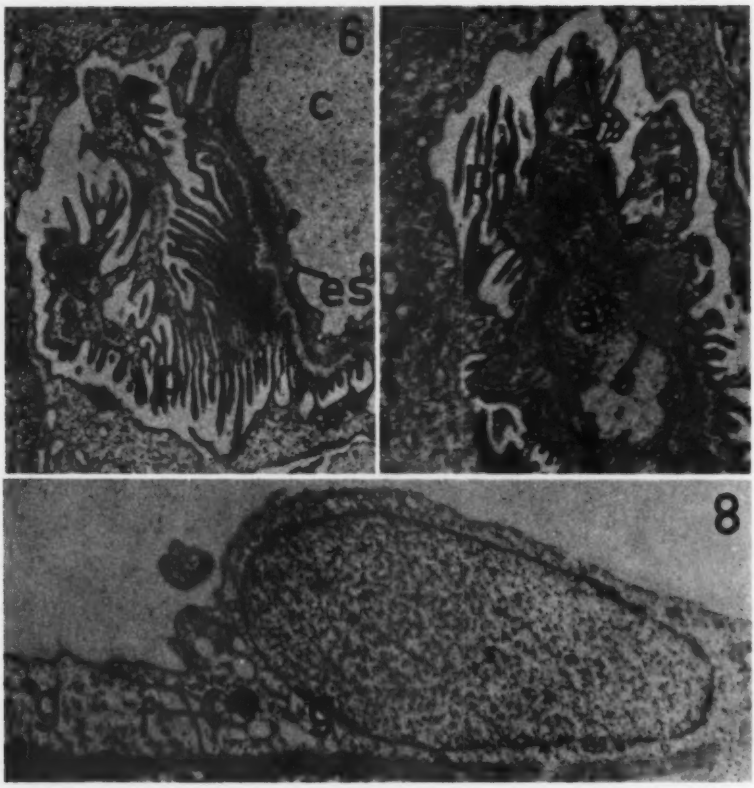

Fig. 6. The micrograph shows a tangential section through a capillary wall and the adjacent pedicels. The complex pattern formed by interdigitating pedicels (p) is clearly visible. The capillary lumen is at (c), the endothelial layer at (es) and the three layers of the basement membrane are clearly seen. × 15,000.

Fig. 7. The micrograph shows a tangential section through the capillary wall with the lamina fenestrata appearance of the endothelial layer at (a). The dense layer of the basement membrane is at (d) and pedicels at (p). × 15,000.

Fig. 8. The micrograph shows the junction (f) of two flattened cells (g) on Bowman's capsular basement membrane (r). × 15,000.

Fig. 9. The micrograph shows a thin section of a glomerulus stained by Jones's silver method. The basement membrane can be clearly seen as a dark line running around capillary loops and investing the glomerulus as a whole. The continuity of Bowman's capsular basement membrane and the basement membrane of the capillary loop can be clearly seen at the hilum.

oblique sections. A similar pattern has been described in higher mammals by Hall (1953a), Pease (1955), and Yamada (1955a).

Intercapillary tissue.

Situated between peripheral capillary loops are cells containing nuclei and projections of cytoplasm which, in the plane of section, are not exposed to blood, from which they are separated by an obvious endothelial cell, while they are separated from Bowman's space by a branch of the capillary loop basement membrane on which rest epithelial pedicels. The intercapillary cell has a large nucleus usually without nucleolus, a moderate amount of cytoplasm containing mitochondria, endoplasmic reticulum and dense granules and shows no obvious morphological difference from the endothelial cell. Policard *et al.* (1955) describe projections of these "mesangial" cells, which push the lining of endothelium before them into the capillary lumen, and later Yamada (1955a) found mesangial projections which he considered had even penetrated the endothelial cytoplasm to become exposed to blood, and he also described a fibrillar element in the cytoplasm of the mesangial cell. I could not verify any of these findings in the opossum. A dense homogeneous material with scattered areas of less dense material fills the intercellular space which in places is extremely wide. A rather similar wide intercellular space is visible in illustrations in the papers of Bergstrand (1957) and Pak Poy and Robertson (1957). However, in the bird, where the intercellular space is narrow, there is often a dense central component (sometimes reduplicated) with less dense layers on the sides. This arrangement was not seen in the marsupial glomerulus. The intercellular material is continuous with the basement membrane of the capillary loop.

Bowman's capsule.

Bowman's capsular basement membrane is composed of a dense homogeneous layer on which rest flattened epithelial cells. Sometimes (probably where slight artefactual separation has occurred) a narrow gap is present between the major dense component and the cytoplasmic membrane of the capsular epithelial cell. These flattened epithelial cells line the basement membrane and do not form a syncytium, for definite cytoplasmic membranes exist where the cells meet. In Fig. 8 such a junction is seen as a dense membrane, which better resolution would no doubt show to be double. The tall columnar epithelial cells described by McNider (1927) in an atypical glomerulus of the American opossum (*Didelphys virginiana*) were not seen.

Hilum.

In this region there is direct continuity between the capillary loop basement membrane, Bowman's capsular basement membrane and the intercellular material of the adventitial coat of the afferent and efferent arterioles. The adventitial coat penetrates into the glomerulus and epithelial pedicels project on to the outer surfaces of the adventitial cells.

DISCUSSION.

Endothelial "pores" have been reported in higher animals by Hall (1953a), Rhodin (1955), Pease (1954, 1955), Yamada (1955a, 1955b) and denied by Rhinehart (1955) who considered the endothelium to be pseudoporous due to vesicles, and Dalton (1951) who regarded the pores as artefact. Similar, though much less numerous, pores have also been noted in the avian glomerulus by Pak Poy and Robertson (1957), who considered that some of them at least may be due to defects in the knife edge. However, the continuity of the outer and inner cytoplasmic membrane at the margins of the pores seen in the present study would suggest that in mice pores may be due to vesicles in the cytoplasm. However, if this were so the defect would be completely surrounded by cytoplasmic membrane irrespective of the plane of sectioning. Undoubtedly these "pores" are definite holes through the thin endothelial sheet, but whether they are true or artefacts produced by shrinkage during fixation of the very thin and widely-spread endothelial cytoplasm appears unlikely to be settled by electron microscopy alone.

The presence or absence in glomeruli of intercapillary tissue other than at the hilum has been a contentious point for many years. Recent electron micrographs of higher mammals by Yamada (1955a), Policard *et al.* (1955) show without any doubt, that in the plane of sectioning there are cells between capillary loops, and that these cells are separated by endothelial cells from direct contact with blood. Pak Poy and Robertson (1957) have shown that the central cell mass of the avian glomerulus is definitely intercapillary irrespective of the plane of sectioning. The large number of intercapillary cells in the bird and the small number of capillary loops in almost every section makes this virtually certain. The central cell mass of the bird is covered by a branch of the capillary loop basement membrane and a similar arrangement is found in the marsupial glomerulus.

Hall (1953a), Mueller *et al.* (1955) have stated that these intercapillary cells in higher mammals are endothelial cells. Hall (1953a) considered that they were tangential sections of a capillary loop lying in another plane. However, if this were so, one would expect to find oblique sectioning of the adjacent epithelial pedicels and the branch of the basement membrane as it passes around the surface of the intercapillary tissue to the next capillary loop. Sections of "intercapillary" tissue in marsupials show a small degree of oblique sectioning in a few areas, but allowing for this and basing conclusions only on perpendicular sections it is not possible to be certain that these cells are truly intercapillary, because the main body of the endothelial cells usually projects some distance into the capillary lumen and spreads to a considerable extent around the circumference, so that a section through this area cutting off an arc would satisfy the above findings in marsupials.

The division of the dense middle layer of the capillary loop basement

membrane was also occasionally seen in the avian glomerulus (unpublished data) and its significance is not known. Jones (1953) and Churg and Grishman (1953) considered the capillary basement membrane to have an epithelial and an endothelial component. Rhinehart *et al.* (1953), Oberling *et al.* (1951), Rhinehart and Farquhar (1954), and Hall *et al.* (1953b), described it as being single in electron micrographs of higher mammals. Gersh and Catchpole (1949) considered basement membrane to be a concentrated form of intercellular ground substance and Fig. 2 shows the direct continuity of intercellular material of the intercapillary tissue with the capillary basement membrane, which, after all, occupies the intercellular space between endothelial and epithelial cells. The ground substance of intercellular material is composed of colloids, diffusible electrolytes and water (Wagner, 1957) and as the cytoplasmic membrane is semipermeable, the ground substance is susceptible to alterations by osmosis, electrolyte disturbance, shrinkage of cells, swelling of cells during fixation, and it would be unwise to apply too much significance to these occasional findings of reduplications and irregularities of the denser lamina of the basement membrane.

In conclusion, the lower mammalian glomerulus has a very similar structure to that of higher mammals.

SUMMARY.

Electron microscopy shows that the renal glomerulus of the opossum (*Trichosurus vulpecula*) has a very similar structure to that of higher mammals. The capillary basement membrane has an outer less dense layer, a middle dense layer and an inner less dense layer and is continuous with the intercellular material of the intercapillary tissue. Lining the capillary lumen is a thin sheet of endothelial cytoplasm which is perforated by numerous "pores". Epithelial cells have the same pedicellar arrangements as in higher mammals. Cells exist between peripheral capillary loops and are separated from direct contact with blood by endothelial cells in the plane of section. No definite conclusion can be reached as to whether they are intra- or intercapillary in position.

Acknowledgments. I am indebted to Professor J. S. Robertson for advice and assistance during this study.

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SERUM CHOLESTEROL AND PHOSPHOLIPID LEVELS OF AUSTRALIAN ABORIGINES[1]

by COLIN J. SCHWARTZ[2], ALLAN J. DAY, J. ANDREW PETERS AND J. R. CASLEY-SMITH

(From the Department of Human Physiology and Pharmacology, University of Adelaide).

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The important rôle of high serum cholesterol levels as a factor in the pathogenesis of atheroma has been extensively studied. There is little doubt that a high and maintained serum cholesterol level is directly related to atheroma both in experimental animals (Day and Wilkinson, 1956), and in humans (Keys and Anderson, 1954). Furthermore, there have been many published reports on the serum lipid levels in different ethnic groups where differences in physical activity, diet or genetic constitution have been invoked to explain variations in the serum lipid levels. These ethnological surveys have shown clearly that groups on a low animal fat diet, have low levels of serum cholesterol, beta-lipoproteins and in some cases, a low incidence of coronary atherosclerotic disease. Walker and Arvidsson (1954), in a study on the South African Bantus, have demonstrated that those who subsist on a low fat diet, have low serum cholesterol levels, while urban Bantus living on a high fat diet have significantly higher cholesterol values. Similar findings for Bantus have been reported by Bronte-Stewart *et al.* (1955), where a low incidence of coronary heart disease has been observed, together with significantly lower levels of total serum cholesterol and cholesterol in the beta-lipoprotein fractions. An investigation on Nigerian natives by Mann, Nicol and Stare (1955), has shown that these people likewise have lower serum cholesterol levels than North Americans. The lower levels are attributed to a low fat intake. Similar results are reported by Mann, Munoz and Scrimshaw (1955), in a study on Central American Indians. Page, Lewis and Gilbert (1956), in an investigation of Navajo Indians, have demonstrated that in this ethnic group, in spite of a normal diet, persistently lower serum lipid levels exist than in a control series of Americans,

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subsisting on an essentially similar diet. It is suggested by these latter workers that the lower lipid levels and the lower incidence of coronary artery disease, many therefore represent a constitutional difference on an ethnic basis.

As no study of the serum lipid levels in Australian aborigines has been undertaken, it was considered worthwhile to determine the serum cholesterol and phospholipid in these people in order to clarify further the factors controlling the levels in humans.

MATERIALS AND METHODS.

Venous blood samples were collected from 90 Australian full-blooded aborigines of varying tribal origins, but mainly Pitjantjaras, Arandas and Pintubis living nomadically in Central Australia in the Haast's Bluff area. Ages were obtained directly wherever possible or assessed by experienced observers, including a dentist. All blood samples were collected in sterile containers, centrifuged as soon as possible, and the sera refrigerated immediately, prior to their despatch to the laboratory by air in vacuum flasks. Controls used for comparison were from individuals of wholly European descent, without any clinical evidence of atherosclerotic disease or other conditions known to affect the serum lipid levels.

Biochemical methods: Total serum cholesterol was determined by the method described by Zlatkis, Zak and Boyle (1953). Phospholipids were determined according to the method of Brown (1954). These methods were modified for use in this laboratory as described in a previous communication by Day, Wilkinson and Schwartz (1956).

Dietary assessment: Food intake, as to the fat, carbohydrate, protein and vitamin content of the bush diet, was assessed on the basis of many years' experience of the eating habits of these people and the naturally occurring foods in the locality under consideration. No detailed caloric values are given. Any supplements obtained from Government rations are small with respect to fat, and are considered in the final assessment.

RESULTS

Diet: The animal fat intake of the Central Australian aborigines from the Haast's Bluff region involved in this present study is decidedly low when compared with the average intake of white Australians. This low intake of fat results both from a scarcity of fat itself, and also from demands made upon available supplies by native customs. It is likely that the males eat more animal fat than the females, because of their readier access to it after hunting, but the difference is probably small. Wichitty grubs (larvae of several species of *Xyleutes* moths) are an important source of fat for both women and children, however. Somewhat less than 10 p.c. of the calories in the aboriginal diet is derived from animal fat, i.e. less than one-third of the calories so derived in the white Australian diet (N. B. Tindale, personal communication).

Serum cholesterol: From Table 1 it can be seen that the mean serum cholesterol values for both white males (263·8) and white females (284·2) are significantly higher than either the aboriginal male (217·0) or female (207·9) means. Further, the mean value for white males increases with age and over 25 years the value (285·9) closely approximates that of the white females (284·2). There is no significant difference between the mean values

TABLE 1.

Serum Cholesterol.

	Aborigines.							Whites						
	$y = 196{\cdot}61 + 1{\cdot}248820x - 0{\cdot}015751988x^2$							$y = 140{\cdot}16 + 6{\cdot}102729x - 0{\cdot}059018917x^2$						
Males	n	$\bar{x}$	$\bar{y}$	S.D.	S.E.			n	$\bar{x}$	$\bar{y}$	S.D.	S.E.		
					$\bar{y}$	bx	bx^2					$\bar{y}$	bx	bx^2
	39	40·026	217·026	37·133	5·946	1·873	0·0203	49	34·571	263·796	46·699	6·671	1·956	0·022585
	$y = 133{\cdot}51 + 4{\cdot}36893x - 0{\cdot}050911914x^2$							No significant regression.						
Females	n	$\bar{x}$	$\bar{y}$	S.D.	S.E.			n	$\bar{x}$	$\bar{y}$	S.D.	S.E.		
					$\bar{y}$	bx	bx^2					$\bar{y}$	—	—
	49	29·612	207·857	26·831	3·833	2·305	0·0200	27	32·926	284·185	47·410	9·124	—	—

Male Whites $\bar{y}(x \leqslant 25) = 240{\cdot}8$

$\bar{y}(x > 25) = 285{\cdot}9$

n = number of subjects.
y = serum cholesterol and quadratic regression equation for cholesterol.
x = age in years.
b = regression coefficient.
S.D. = Standard deviation.
S.E. = Stardard error.

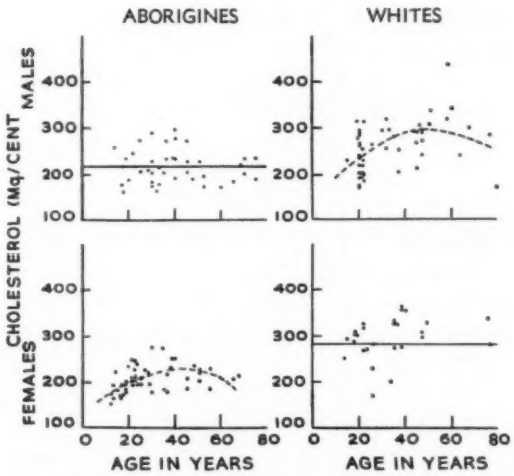

Fig. 1. Individual serum cholesterol values are plotted around the means (straight lines) or the curves (interrupted lines) derived from quadratic regression equations. The effect of age changes can be readily seen.

for aboriginal male (217·0) and aboriginal female (207·9). Fig. 1 shows the distribution of individual values around the curves for cholesterol as derived from the quadratic regression equations given in Table 1. There is no significant regression for age in the aboriginal male, but for the aboriginal female the regression for age is statistically significant ($p < 0{\cdot}01$). For this latter group the mean levels rise to a maximum at 40 years and thereafter decline gradually. The ageing regression for white males is also significant ($p < 0{\cdot}01$). In this group the mean levels for age rise until the fifth decade and then slowly decline. There is no significant regression for age, either quadratic or linear, in the case of the white females.

Phospholipids: Table 2 summarizes the data for serum phospholipids. As there is no significant regression for ageing the individual values are plotted around the means, as shown in Fig. 2. The mean phospholipid level for the aboriginal females (232·1) is significantly higher than the mean for aboriginal males (205·6). The aboriginal females also show a mean level (232·1) which is significantly higher ($p < 0{\cdot}001$) than the mean for white females (196·9), but the difference between aboriginal male and white male is not significant, nor is the difference between white male and white female. There is no significant change due to age in either aborigines or whites, according to an analysis of both quadratic and linear regression values.

Cholesterol-phospholipid ratio: A summary of data is presented in Table 3. From this it can be seen that the mean values for both white males and females are significantly higher than the means for either aboriginal males or females. The mean value for white females (1·450) is also significantly higher than the mean for white males (1·248) while the aboriginal male mean (1·064) is higher than the aboriginal female mean (0·906). These differences are highly significant ($p < 0{\cdot}001$). Fig. 3 depicts the distribution of individual values around the curves for quadratic regression values or the mean, as in the case

TABLE 2.
Serum Phospholipid.

	Aborigines					Whites				
	n	$\bar{x}$	$\bar{z}$	S.D.	S.E.	n	$\bar{x}$	$\bar{z}$	S.D.	S.E.
Males	39	40·026	205·641	39·400	6·309	49	34·571	212·857	40·380	5·769
	n	$\bar{x}$	$\bar{z}$	S.D.	S.E.	n	$\bar{x}$	$\bar{z}$	S.D.	S.E.
Females	49	29·612	232·163	40·690	5·813	27	32·926	196·926	34·142	6·571

z = Serum phospholipid.

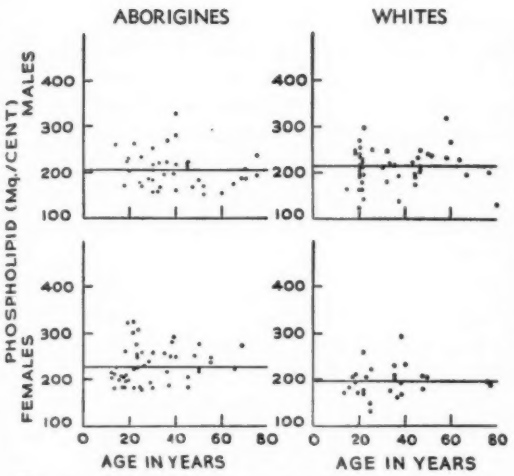

Fig. 2. Individual serum phospholipid values are plotted around the means. No changes due to age are apparent.

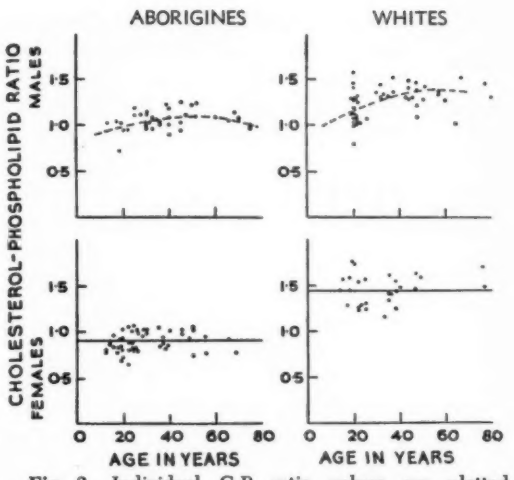

Fig. 3. Individual C-P ratio values are plotted around either the means (straight lines) or the quadratic regression curves. The effect of age changes and differences between groups are readily seen.

TABLE 3.

Cholesterol-Phospholipid Ratio.

	Aborigines.							Whites						
	$w = 0{\cdot}77825 + 0{\cdot}013156x - 0{\cdot}00012828x^2$							$w = 0{\cdot}90905 + 0{\cdot}015251451x - 0{\cdot}000127238x^2$						
Males	n	$\bar{x}$	$\bar{w}$	S.D.	S.E.			n	$\bar{x}$	$\bar{w}$	S.D.	S.E.		
					$\bar{w}$	bx	bx^2					$\bar{w}$	bx	bx^2
	39	40·026	1·064	0·096	0·015	0·0049	0·000053	49	34·571	1·248	0·1400	0·020	0·00586	0·000068
	$w = 0{\cdot}723000 + 0{\cdot}011726x - 0{\cdot}000152131x^2$							No significant regression						
Females	n	$\bar{x}$	$\bar{w}$	S.D.	S.E.			n	$\bar{x}$	$\bar{w}$	S.D.	S.E.		
					$\bar{w}$	bx	bx^2					$\bar{w}$	bx	bx^2
	49	29·612	0·906	0·105	0·015	0·0051	0·000067	27	32·926	1·450	0·1586	0·0305	—	—

w = C-P Ratio and quadratic regression equation for C-P Ratio.

of white females where there is no significant regression for age. The regression for age in aboriginal males is significant ($p < 0{\cdot}05$) the values rise to a maximum at 50 years and then decline. There is no significant ageing change for either aboriginal or white females, but in the case of white males both linear and quadratic regressions are highly significant ($p < 0{\cdot}001$). In this latter group the C-P ratio rises with ageing and is maximal at 60 years after which the values fall.

DISCUSSION.

The serum cholesterol levels of white Australians, both male and female, have been shown to be significantly higher than those for aborigines. The dietary intake of fat by the aborigine is very much lower than in the normal white Australian diet. It is probable therefore that the low serum cholesterol levels result from this lower dietary intake of animal fat. Similar findings are reported on the Bantus by Bronte-Stewart *et al.* (1955) and Walker and Arvidsson (1954), where the quantity of dietary animal fat has been specifically implicated as a factor controlling the serum cholesterol levels in man. It has recently been shown that animal fat feeding in humans will produce an increase in the serum cholesterol level, while a fat free diet will reduce the serum cholesterol levels (Beveridge *et al.*, 1956; Hatch *et al.*, 1955).

Not only the quantity of ingested fat, but also the quality, appears to influence the cholesterol level in humans. Certain plant fats reduce cholesterol levels (Beveridge *et al.*, 1956; Ahrens *et al.*, 1955). A similar effect has been shown with vegetable fat by Kinsell *et al.* (1952). These workers have, in addition, shown a decrease in phospholipid with vegetable fat feeding.

The significance of the effects of vegetable fats upon the serum cholesterol levels in the present study is uncertain. It is possible, however, that the aborigines who subsist mainly on a lean meat, high vegetable diet, may reflect in their cholesterol levels the depressant action of vegetable fats, in addition to the more obvious effect of a low animal fat intake. Any effect on the serum levels from ingested cholesterol is unlikely as the serum level in humans has been shown to be independent of cholesterol intake (Keys *et al.*, 1955, 1956).

In this present work a significant increase in the serum cholesterol levels of white males with ageing is demonstrated, the peak occurring at 50 years, after which the levels slowly decline. This is in accordance with the age changes reported by others.

Sharply contrasted is the absence of any such ageing increase in the aboriginal males. This absence has been observed in other ethnic groups (Page *et al.*, 1956; Mann *et al.*, 1955a; Mann *et al.*, 1955b). The white females described here show no increase with age, while in the aboriginal females such an increase is apparent.

Factors affecting the value of serum phospholipid levels are not well understood. A significant sex difference in the phospholipid levels is to be noted in the aborigines, the females having significantly higher values than the males. This is not readily explained. Oestrogen changes are an unlikely explanation, as they have been shown by Oliver and Boyd (1954) to have no effect on serum phospholipid levels.

The cholesterol-phospholipid ratios of the controls have been shown to be significantly higher than those for the aborigines. This difference is essentially due to the higher cholesterol levels in the former groups. In the aboriginal female, the C-P ratio is significantly lower than that of the male. This reflects the difference in phospholipid levels mentioned earlier.

As an index of atherogenicity, the C-P ratio has received considerable attention. It has been shown to be elevated in patients suffering from atherosclerotic heart disease (Morrison, 1952). Whether this elevated C-P ratio is real or apparent is another question. Possibly it is elevated merely because of elevated serum cholesterol and this latter factor represents the true atherogenetic index. That this is so in experimental animals has been demonstrated by Day and Wilkinson (1956).

Unfortunately, in the present study no correlation of the serum lipids with the incidence of atheroma in aborigines is possible owing to a paucity of autopsy and clinical data.

SUMMARY.

The serum cholesterol level of Australian aborigines has been shown to be significantly lower than that of Australian white controls.

No elevation of serum cholesterol level in aborigines with ageing has been observed. This is contrasted with the increase shown as a result of ageing in the white controls.

Aboriginal female phospholipid levels have been shown to be significantly higher than the levels for white females, and also for aboriginal males. The C-P ratios for aborigines are significantly lower than those in the controls.

The significance of these findings is discussed in terms of the lower intake of fat of the aborigines.

Acknowledgments. We are pleased to record our indebtedness to Professor A. A. Abbie, leader of the expedition, for his cooperation and helpful advice. We also thank Mr. G. N. Wilkinson of the Division of Mathematical Statistics, C.S.I.R.O., for the analysis of data, and Mr. N. B. Tindale of the S.A. Museum for assessing ages and diet.

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SERUM LIPID RESPONSES TO HEPARIN AND TO PROTAMINE IN RABBITS

by ALLAN J. DAY, COLIN J. SCHWARTZ,[1] AND J. ANDREW PETERS

(From the Department of Human Physiology and Pharmacology, University of Adelaide).

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The daily administration of Heparin Retard has been shown to lower the serum total fatty acids, phospholipids and β-lipoproteins in cholesterol-fed rabbits (Day *et al.*, 1957a). Others workers, however, have failed to obtain changes produced by heparin in this species when visible turbidity or ultracentrifuge patterns have been used as an index (Nikkilä, 1953; Graham and Schumaker, 1956). Comfort (1953, 1955) suggests that the injection of heparin gives rise to several different activities and that these may vary independently from species to species. He has shown that heparin in rabbits, while causing an increase in the electrophoretic migration rate of β-lipoprotein, does not produce clearing of visible lipemia. This failure to clear visible lipemia or to alter lipoprotein levels is attributed by him not to a lack of clearing factor elaborated, but to a resistance of rabbit lipemia to its action (Comfort, 1953, 1955). The effect of heparin in changing the physical state of the blood lipids is not the only result of its administration and in other species in addition to these changes, heparin has been shown to cause alterations in the chemical content of the serum lipids and in particular the fatty acids (Brown, 1952; Grossman *et al.*, 1954; Day *et al.*, 1957b). It is possible that the chemical changes induced by heparin may be distinct from the physical effect on the distribution of lipids. In view of the definite effects cited above of heparin on lipids in cholesterol-fed rabbits (Day *et al.*, 1957a) the possibility arises as to whether there is a dissociation of these two effects in this species. The effect of heparin on the serum lipids in rabbits has therefore been further investigated and the serum lipid changes occurring immediately after the intravenous injection of heparin determined, both in normally fed rabbits, and in rabbits with varying degrees of lipemia induced by cholesterol feeding.

Further, in view of the elevation produced by toluidine blue on the serum lipids in rabbits (Day *et al*, 1956, 1957a) and its possible significance with regard to the physiological action of heparin in lipid transport, it was decided

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to determine the effect of the heparin-antagonist protamine on serum lipids in the rabbit. If the toluidine blue effect was a manifestation of its anti-heparin action, as was suggested, then protamine might be expected to have a similar effect on the blood lipids. The effect of protamine was therefore compared with that of heparin under similar circumstances and at varying degrees of lipemia induced by cholesterol feeding.

METHODS.

Eighteen rabbits of mongrel stock and mixed sexes (1·3 kg. to 2·2 kg.) were investigated. These rabbits were divided into three groups with six in each and each animal was used on three separate occasions. The rabbits were fasted overnight and two blood samples 15 minutes apart were taken. Following the second sample 1000 units of heparin (in the case of the first group) were administered intravenously, and two further blood samples removed 20 minutes and 60 minutes following the injection. The second group was treated similarly as regards sampling, but instead of heparin 10 mg. protamine sulphate were administered intravenously. The third group of six rabbits served as a control and received 3 ml. of saline intravenously with blood samples before and after as for the first two groups. The three groups were then given a cholesterol supplemented diet containing daily, 1 gm. cholesterol and 6 ml. peanut oil in 75 gm. rabbit mash. The effects of heparin, protamine and of saline were determined again after three days cholesterol feeding and on a third occasion after twenty-eight days cholesterol feeding. The procedure as regards sampling, etc., was the same as for the fasting state, the first group again receiving heparin, the second protamine and the third saline. The blood samples collected were centrifuged at 3,500 r.p.m. for 15 minutes, the serum removed and serum cholesterol, serum phospholipid and serum total esterified fatty acids determined in each sample. Thus the effect of both heparin and of protamine on the serum lipids at varying levels of lipemia, was studied.

Serum lipids – Serum cholesterol was determined by the method of Zlatkis, Zak and Boyle (1953), serum phospholipid by the method of Brown (1954), and total fatty acids by the method of Stern and Shapiro (1953). These methods were modified as already described (Day *et al.*, 1956).

Statistical analysis of data – The scatter diagrams indicated that the response to injection in any serum fraction and likewise the variation in response was more or less proportional to the level of the serum fraction before injection. In this situation the appropriate statistical analysis is on the logarithmic transforms of the serum measurements. On a logarithmic scale of measurements the proportional effects become more or less constant and the variation is rendered homogeneous. Analysis was carried out therefore of the effect of heparin and of protamine on the serum lipids at the three phases of lipemia, the criteria analysed being the logarithms of the ratio of the mean serum level of the respective lipid fraction after injection to that before injection. Differences between phases in *proportionate* response to each of the three treatments (heparin, protamine and saline) were then examined by analyses of variance.

RESULTS.

The effect of heparin, protamine and of saline on the serum total esterified fatty acid levels is shown in Fig. 1. The rise or fall is plotted against the mean pre-injection level, the three phases (fasting, 3 days cholesterol and 28 days cholesterol) being considered together according to their initial lipid level.

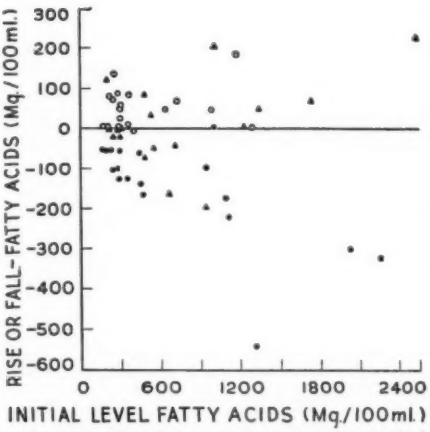

Fig. 1. The change in serum total esterified fatty acids produced by heparin, protamine and saline at different levels of lipemia. ● Heparin. △ Saline. ○ Protamine. Details in text.

Heparin produces a definite fall in total fatty acids, a fall which can be seen to be proportionate to the level existing before the injection. The magnitude of the reduction due to heparin is tabulated in Table 1. It can be observed that considering all levels of lipemia, heparin produced a net fall in total fatty acids amounting to 27·2 p.c. Table 1 also gives the statistical data, i.e., the logarithm of the ratio of the serum level after heparin to that before heparin, together with its estimated standard error. From these data it is possible to see that heparin produces a significant effect on the fatty acids in rabbits at all levels of lipemia. The effect of heparin in reducing fatty acids compared with the effect of saline is very highly significant ($p < \cdot 001$). Analysis of variance between the 3 phases of the experiment revealed no significant difference in proportionate effect between phases.

Protamine produces just the reverse changes to heparin on the total fatty acids. Fig. 1 demonstrates the rise in level produced by this substance at all levels of lipemia. The proportionate effect following protamine was not nearly as marked as that following heparin administration. In fact, it can be seen from Table 1 that the proportionate effect has fallen off with increasing lipemia being 21·6 p.c. in the fasting state and 15·4 p.c. and 5·7 p.c. at 3 and 28 days feeding respectively, i.e., it would appear that the protamine effect is an absolute one and not proportionate to the level. However, this change in proportionate effect was not significant although suggestive of a trend. Table 1 confirms the statistical significance of the general protamine effect on fatty acids the net effect being a 14·0 p.c. rise, which compared with the saline control group, is highly significant ($p < \cdot 01$).

The changes in serum cholesterol produced by heparin, protamine and saline are recorded in Fig. 2. The rise or fall in cholesterol level is plotted against the initial level. Heparin produces a marked fall at all levels of lipemia, an effect which can be seen to increase as the level of serum cholesterol increases. The net fall in cholesterol produced by heparin amounted to 14·7 p.c. and the percentage fall, together with the other relevant statistical information in the various phases, is included in Table 1. The cholesterol determinations

in the fasting animals were unsatisfactory owing to technical difficulties and unfortunately the information at this phase is not available, only two phases being represented with respect to cholesterol. The effect of heparin on serum cholesterol can be seen to be significant at both phases reported and when the pooled data are considered the effect of heparin compared with saline is highly significant ($p < \cdot001$). The effect of protamine on the serum cholesterol is practically a mirror image of the effect of heparin. Protamine produced a rise in level amounting to 10·7 p.c. when the pooled data are considered, a rise which is highly significant ($p < \cdot001$). This effect of protamine can be observed from Fig. 2, while the detailed statistical information can be obtained from Table 1.

TABLE 1.

The effect of heparin, protamine and saline on the percentage rise or fall in serum lipids and on the logarithm of the ratio of lipid level after injection to that before injection (with its standard error) at various levels of lipemia.

	Treatment	Fasting	3 days cholesterol	28 days cholesterol	Pooled results
Total Fatty Acids	Heparin	*−32·7* p.c. −0·172±0·021 (6)*	*−29·5* p.c. −0·152±0·034 (6)	*−18·7* p.c. −0·090±0·032 (6)	*−27·2* p.c. −0·138±0·017 (18)
	Protamine	*21·6* p.c. 0·085±0·032 (6)	*15·4* p.c. 0·062±0·018 (6)	*5·7* p.c. 0·024±0·011 (6)	*14·0* p.c. 0·057±0·013 (18)
	Saline	*4·5* p.c. 0·019±0·037 (6)	*−7·1* p.c. −0·032±0·028 (6)	*2·1* p.c. 0·009±0·026 (6)	*−0·5* p.c. −0·002±0·018 (18)
Cholesterol	Heparin		*−10·9* p.c. −0·050±0·014 (6)	*−18·5* p.c. −0·089±0·010 (6)	*−14·7* p.c. −0·069±0·008 (12)
	Protamine		*16·8* p.c. 0·067±0·027 (6)	*4·7* p.c. 0·020±0·007 (6)	*10·7* p.c. 0·044±0·014 (12)
	Saline		*−4·3* p.c. −0·019±0·006 (6)	*−4·5* p.c. −0·020±0·005 (6)	*−4·5* p.c. −0·020±0·004 (12)
Phospholipid	Heparin	*−10·7* p.c. −0·049±0·015 (6)	*−10·3* p.c. −0·047±0·012 (6)	*−12·1* p.c. −0·056±0·009 (6)	*−11·1* p.c. −0·051±0·007 (18)
	Protamine	*3·0* p.c. 0·013±0·014 (6)	*2·1* p.c. 0·009±0·014 (6)	*4·5* p.c. 0·019±0·013 (6)	*3·3* p.c. 0·014±0·008 (18)
	Saline	*−8·0* p.c. −0·036±0·017 (6)	*−1·4* p.c. −0·006±0·021 (6)	*−1·8* p.c. −0·008±0·006 (6)	*−3·6* p.c. −0·016±0·009 (18)

* Number of animals.

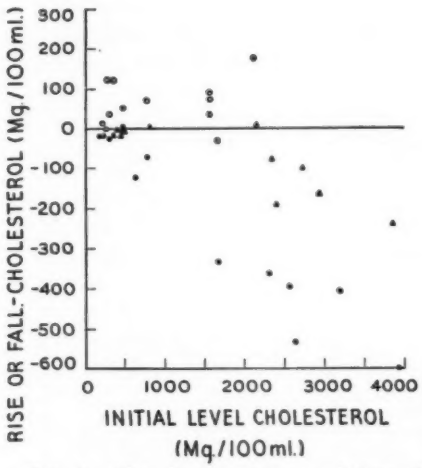

Fig. 2. The change in serum cholesterol produced by heparin, protamine and saline at different levels of lipemia. ● Heparin. △ Saline. ○ Protamine. Details in text.

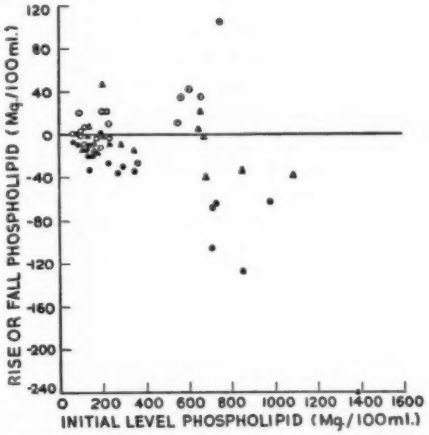

Fig. 3. The change in serum phospholipid produced by heparin, protamine and saline at different levels of lipemia. ● Heparin. △ Saline. ○ Protamine. Details in text.

One further aspect of the changes in serum cholesterol needs comment. Following 3 days' feeding, heparin produced a fall in serum cholesterol amounting to 10·9 p.c., while at 28 days' feeding a greater proportionate fall was produced (18·5 p.c.). Analysis of variance demonstrates that this increased proportionate fall at higher levels is significant ($p < \cdot 05$). The reverse picture occurred with protamine the rise being 16·8 p.c. at 3 days and 4·7 p.c. at 28 days and while this change is non-significant, it might be argued that this is due to the abnormally higher standard error in the 3 days determinations and that a real falling off in proportionate effect has occurred in the protamine-treated group.

Fig. 3 gives the effect of heparin, protamine and saline on the serum phospholipid at the various levels of lipemia. Again the rise or fall in level is plotted against the initial level prior to the injection. The proportionate rise or fall in level at the three phases of lipemia is shown in Table 1, which also includes the statistical data. Heparin produces a fall in phospholipid level, which is proportional to the initial level. Thus at the three phases, falls of 10·7 p.c., 10·3 p.c. and 12·1 p.c. were recorded, and the net fall considering the pooled data was 11·1 p.c. This effect of heparin in reducing the phospholipid is highly significant ($p < \cdot 01$). Protamine again produced an effect opposite to that of heparin, a rise occurring at all levels. This rise was more marked than is apparent at first sight if one considers the small dilution effect (i.e., following fluid exchange with bleeding), which shows up in all the saline data. In any

case, protamine produced a significant rise ($p < \cdot 01$) in phospholipid level when compared with saline. The effect on phospholipid is apparently proportional to the initial level this being more apparent (Fig. 3) at the higher levels of lipemia than in the fasting specimens.

DISCUSSION.

Further evidence has been produced in this paper that heparin causes chemical changes in the serum lipids and that these changes can be brought about in the rabbit. Heparin has been shown to produce a reduction in the serum total fatty acid, phospholipid and cholesterol levels. The mechanism of the clearing of induced lipemia by heparin has been interpreted in terms of lipolysis of triglycerides and removal of the free fatty acids liberated (Nichols *et al.*, 1952; Grossman *et al.*, 1954; Robinson and French, 1953). This aspect is valid in explanation of the rabbit responses as has been suggested (Day *et al.*, 1957a). The interesting point brought out in this present paper is the dependence of the effect of heparin on the initial level of lipemia. The magnitude of the fall produced by heparin in the fasting state is not great, but as the lipemia increases with progressive cholesterol feeding the absolute effect of heparin increases considerably in the case of all the lipid fractions studied. A much more constant picture of the lipid responses is therefore given by considering proportionate effects. This increasing influence of heparin at higher lipid levels is particularly marked in the case of the cholesterol and phospholipid, where the changes are quite small at lower levels of lipemia. If these had been considered exclusively it might have been impossible to detect the changes produced by heparin in these two fractions, changes which show up quite definitely at higher levels of lipemia. This observation of an increasing effect of heparin with increasing level of lipemia may be relevant in explaining the apparent contradictory effects described in human subjects. It is significant as regards its action on serum cholesterol, that where individuals with low levels of cholesterol are used, heparin fails to produce a reduction (Lever *et al.*, 1953; Day *et al.*, 1957b). Where the effect has been studied in patients with pathologically high serum cholesterol levels, heparin has been shown to have significant effects on this fraction (Lever *et al.*, 1953; Basu and Stewart, 1950). It is possible that these differing responses are due to the increased effect of heparin on blood cholesterol at higher lipid levels as has been shown for rabbits. This matter is highlighted still further by the observation made here that not only is the absolute effect of heparin on serum cholesterol increased at higher lipid levels, but the proportionate effect with respect to this fraction is also increased. Thus a 10·9 p.c. fall occurred in the animals fed cholesterol for 3 days, and an 18·5 p.c. fall in those fed cholesterol for 28 days, this difference between phases being statistically significant. It would seem that as far as the effect of heparin on cholesterol at least is concerned this effect is sensitised by increasing lipid levels.

As regards the fatty acid changes, these are distinct at all levels of lipemia, even in the fasting state. As has already been pointed out (Day *et al.*, 1957a) where fatty acid measurements are made as an index of heparin action, definite effects have been recorded. However, even with the fatty acids a marked increase in absolute effect occurs with increasing lipemia, and while the percentage effect tends to be reduced at higher levels (see Table 1) it is not sufficient to show any statistically significant difference between the 3 phases. Thus heparin produces changes in serum lipids which are increased in proportion to the level of lipemia, and in the case of the cholesterol fraction increased even more than in proportion to that level as the latter is elevated. This sensitising effect to heparin at higher lipid levels has been observed, also by Comfort (1954) with regard to the effect of heparin on β-lipoprotein migration. This worker finds minimal effects on this migration below cholesterol levels of 1 mg./ml. but appreciable effects above this level.

Concerning the effect of heparin in the rabbit in particular, it appears definite that this substance does cause changes in this species, and while it may not produce clearing of visible lipemia or changes in ultracentrifugal patterns (Nikkilä, 1953; Schumaker, 1956; Anfinsen *et al.*, 1952; Comfort, 1953, 1955) it does cause a fall in content of serum lipids as determined by chemical methods. The possible explanation in terms of a dissociation of heparin effects has been already outlined and it is possible that the resistance of rabbit lipemia to redistribution of lipids and consequent visible clearing is not shared by the invisible serum lipid content and that changes in the latter in response to heparin may occur independently.

The increase in lipid levels produced by protamine in this investigation strongly supports the explanation that lipid changes produced by toluidine blue in the rabbit are due to its antiheparin action. It might be expected that if the increase in serum lipids produced by toluidine blue were due to its toxicity or some other action apart from its antiheparin action, that protamine would not cause similar effects. That it does cause similar effects has been established, and it is reasonable to assert that these changes, which are opposite to those produced by heparin under similar circumstances are due to an elimination of circulating heparin in the rabbit and a prevention of its normal rôle of aiding lipid transport. This aspect has been rather more fully dealt with previously (Day *et al.*, 1956, 1957a).

One further point concerning protamine action requires additional explanation. It will be noted that although protamine produces an action opposite to that of heparin in many respects, it does not do so convincingly as regards the increased effect at higher levels of lipemia. While the effect of protamine on phospholipid increases in proportion to the initial lipid level, that on the cholesterol and fatty acid level does not increase in proportion to the initial level, but remains of the same order throughout. One might expect that if

heparin effects were greater at higher lipid levels then elimination of a constant amount of heparin by protamine would result in a correspondingly greater effect. The amount of heparin present in the circulation is certainly very small and this is particularly so with the rabbit. The amount present in the circulation may therefore have more than sufficient lipid "substrate" to deal with in the fasting state. Thus the low amount of heparin may be a limiting factor and higher levels of lipemia are consequently not affected any more than lower levels. With the higher doses of heparin administered there is presumably adequate present to deal with large amounts of lipid and therefore if this is presented to it, as it is with lipemic serum, a proportionately greater effect will occur. The proportionate effect of heparin may thus be looked upon in terms of an increasing amount of a limiting substrate rather than as a sensitisation to a dose of heparin. The failure to increase proportionately in the case of protamine may correspondingly be interpreted in terms of a limiting physiological supply of heparin.

SUMMARY.

The effect of intravenous administration of heparin and of protamine on the serum total esterified fatty acids, serum cholesterol and serum phospholipid in rabbits in the fasting state was determined. This effect was compared with that in rabbits with varying degrees of lipemia induced by cholesterol feeding.

Heparin produced a highly significant reduction in the level of all three lipid fractions studied. The absolute effect of heparin increased in proportion to the level of lipemia existing prior to the injection. This increase in effect was proportional to the initial level in the case of the fatty acids and phospholipids, but in the case of the cholesterol the percentage effect was even greater at higher levels of lipemia.

Protamine produced a highly significant elevation in the level of all three lipid fractions. The increase in absolute effect at higher lipid levels was not nearly so marked as was the case with heparin and no true proportional effect with increasing levels of lipemia was apparent.

The significance of these findings in terms of the action of heparin on blood lipids and of the physiological rôle of heparin in fat transport in the rabbit is discussed.

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TOXOPLASMA IN QUEENSLAND.

I. OBSERVATIONS ON A STRAIN OF *TOXOPLASMA GONDII* ISOLATED FROM A BANDICOOT, *THYLACIS OBESULUS*

by J. H. POPE, E. H. DERRICK AND I. COOK

(From the Queensland Institute of Medical Research, Brisbane).

(*Accepted for publication 24th July, 1957.*)

Toxoplasma gondii (Nicolle and Manceaux, 1908) is a protozoon with a wide host range. It is pathogenic for man as well as for various other species of mammals and birds. From the sporadic human and animal cases that occur, it is evident that *Toxoplasma* is endemic in many countries, and this is further supported by the reports of a high proportion of positive dye and skin tests in humans. A number of epidemics in mammals and birds have been attributed to *Toxoplasma*. Adequate reviews of toxoplasmosis have appeared in recent years, as, for instance, the symposium in 1953 in the American Journal of Tropical Medicine and Hygiene.

A new host of *Toxoplasma* is recorded in the present paper, with details of the observations made, as it appears to be the first Australian strain to be investigated at all fully.

MATERIALS AND METHODS.

Isolation of the B316 Strain of Toxoplasma.

Bandicoot 316, from which the strain was isolated, was trapped at Taringa, a suburb of Brisbane, on February 1, 1951. It was a female *Thylacis obesulus* (Shaw and Nodder),[1] weighing 1106 gm., with seven young in the pouch. At autopsy, the spleen was found to be 1·7 p.c. of the body weight. This indicated gross splenomegaly, according to the observations of Derrick *et al.* (1939). Many small nodules were noted in the lungs, and others were attached to the under surface of the liver.

A saline suspension of liver, spleen and brain was inoculated intraperitoneally into two weaned mice. These were examined, and passaged, at seven days; one had slight splenomegaly. Both second passage mice, and the majority in subsequent passages, showed definite splenomegaly, and it was evident that an infective agent had been transmitted to the mice. Toxoplasms were first demonstrated in the lung of a fourth passage mouse.

The bandicoot suspension was also inoculated into two weaned mice intracerebrally and six two-day-old mice intraperitoneally. Splenomegaly appeared on passage in each instance, but these lines were discontinued after four and three passages respectively, as the splenomegaly was less consistent than with the intraperitoneal route in weaned mice.

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[1] Syn. *Isoodon torosus* (Ramsay).

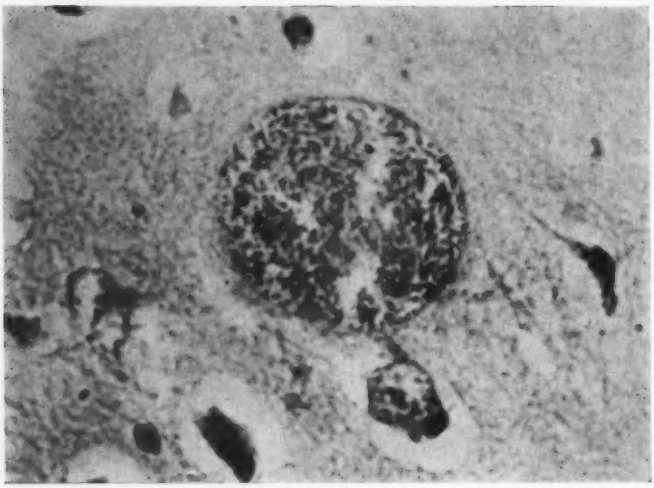

Fig. 1. Pseudocyst in section of brain of bandicoot 316. H. & E. × 780.

Two guinea-pigs also were inoculated with the suspension, but no sign of infection appeared in them nor in a second subinoculated pair.

When tissue sections of Bandicoot 316 were examined, pseudocysts resembling those of *Toxoplasma* were found in the brain (Fig. 1). Their presence provided strong evidence that the strain being passaged in the mice was derived from the bandicoot and not from the mouse stock. There was a little scattered infiltration of cells in perivascular spaces and meninges, indicating a slight degree of encephalitis. There was no cellular reaction around the pseudocysts. The nodules in the lungs were of chronic inflammatory nature; in some areas there was some histological resemblance to the toxoplasmic pneumonitis observed later in our experimental mice; other areas were quite different, although of undetermined etiology. The nodules attached to the liver did not appear to be toxoplasmic in origin. No toxoplasms were found, in spite of prolonged search, in sections of lung, liver, spleen, kidney and retroperitoneal lymph node.

Behaviour of the Strain while Relatively Avirulent for Mice.

The strain was maintained in weaned mice by intraperitoneal injections, usually of a liver-spleen emulsion, at intervals of 14 to 28 days. By October, 1954, it had completed 64 passages. During this phase, the parasite was only slightly virulent for mice. Infected animals appeared well, and the mortality ascribable to the infection was nil.

At autopsy, splenomegaly was the most consistent finding. It was occasionally present seven days after inoculation, usually at 14 days, and was maximal at three to four weeks. The spleen index (product of length and breadth in millimetres) of 78 mice observed at 21 days ranged from 80 to 225 with a mean of 137. (The mean index in 100 mice examined after inoculation with

presumably non-infective material was 80, with a standard deviation of 19.) The spleen was enlarged in nearly all mice examined from five to 24 weeks after inoculation, but was within normal limits in eight mice from 28 to 52 weeks. Other findings in the mice were minute nodules in the lungs and very occasional ascites.

Infection was also successfully transferred by intracerebral and by intranasal inoculation.

The one attempt made to infect mice by feeding infected tissues failed. Livers and spleens, shown to be infective by subinoculation, were ground, mixed with crushed mouse cubes, and offered to 12 mice. All but one had eaten their portions within 3½ hours. No mice showed signs of infection, and two blind passages from six of them were negative.

Infection was not transferred by contact. Twenty uninoculated mice were housed in the same tins as infected mice, and were examined between 29 and 420 days later. Except that the spleens of three were slightly enlarged, they showed no sign of infection. Tissues of these three and four others were subinoculated with negative results.

Histo-pathology.—The most striking lesions were found in the lungs, which showed focal interstitial pneumonitis (Fig. 2). Two weeks after inoculation, small, scattered foci of large mononuclear cells could be seen in the interalveolar septa. At three and four weeks, these were larger and more numerous, and the

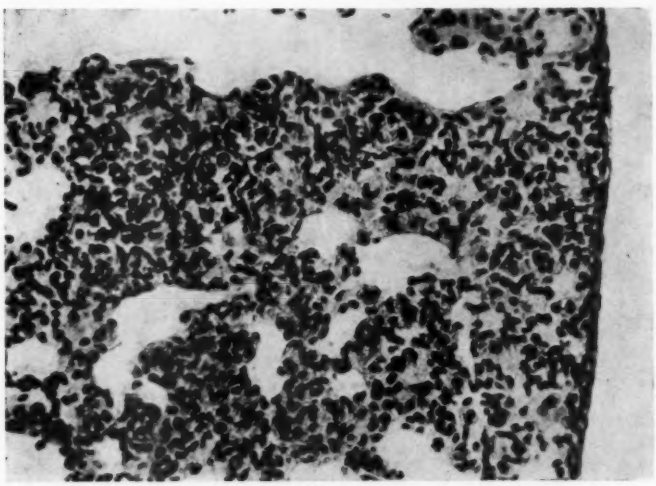

Fig. 2. Lung of mouse MQ1266, 30 days after inoculation with the avirulent phase of the B316 strain. Interstitial pneumonitis, due to the accumulation of large mononuclear cells in the septa. H. & E. × 280.

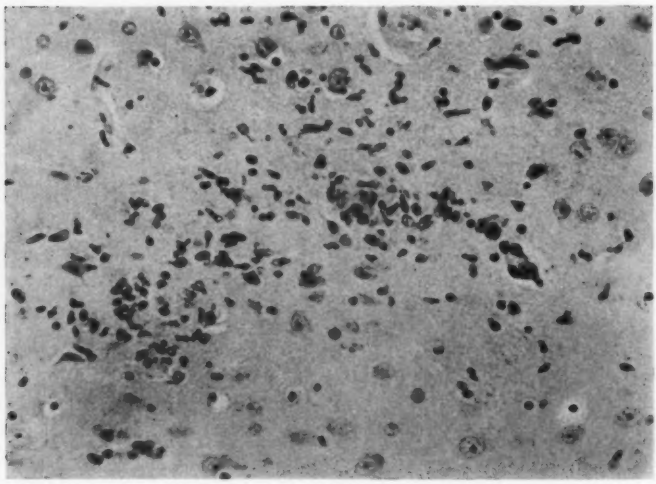

Fig. 3. Inflammatory focus in brain of mouse MQ1267, 36 days after inoculation with the avirulent phase. H. & E. × 280.

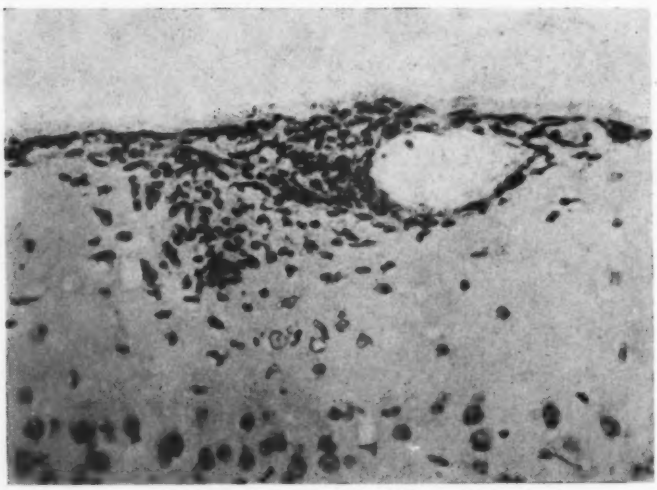

Fig. 4. Meningeal focus in mouse MQ1265, 22 days after inoculation with the avirulent phase. H. & E. × 280.

thickened septa formed a widespread network, or coalesced by collapse of alveoli to form irregular nodules up to 250 μ across. There was no necrosis or fibrosis, and no cellular exudate into the alveoli. During the second month, the inflammatory process usually resolved, although interstitial foci of mononuclear cells were occasionally seen afterwards. Toxoplasms were very scanty. An occasional group was found after careful search in lung sections of three of six mice examined 20 to 30 days after inoculation; they were not found in lungs of 19 mice examined from 35 to 533 days.

In the brain, a few inflammatory lesions were present in practically every mouse examined from 14 to 533 days after inoculation. These included foci of inflammatory cells in the brain substance, as illustrated in Fig. 3 (these were not seen after 168 days), perivascular cuffing, and meningeal foci consisting mainly of lymphocytes (Fig. 4). Calcification was seen in one mouse at 533 days. Pseudocysts were seen in smears or sections of the brains of 11 of 36 mice examined from 7 to 533 days after inoculation; they were first seen at 35 days.

Hyperplasia of both the Malpighian bodies and the red pulp contributed to the enlargement of the spleen. A few small foci of inflammation or degeneration were often noted in liver and heart and occasionally in thigh muscle; their relation to *Toxoplasma* is doubtful, as similar foci may be seen in stock mice. Small inflammatory foci were often observed in kidney and rarely in pancreas.

Distribution and persistence in tissues.—This was studied in the various organs of 28 mice inoculated intraperitoneally. They were drawn from the fourth to the tenth passages, and were examined at intervals from one to 76 weeks after inoculation. Infectivity of organs was assessed by the presence of splenomegaly in subinoculated mice.

Three mice were inoculated with each tissue tested, and examined three weeks later. In comparison with the control mice already mentioned, a mean spleen index exceeding 99 in a group of three mice would be significantly raised ($P = 0{\cdot}05$) and was accepted as presumptive evidence that the inoculum was infective. In many cases the spleen index greatly exceeded 99 and, in Table 1, degrees of splenomegaly are indicated by $^{+}$, $^{++}$ and $^{+++}$. The highest individual index was 228, and the highest mean of three 185.

The results in Table 1 show that the distribution of toxoplasms varied considerably in individual mice. In general, two stages may be seen in the course of the infection.

In the first or parasitaemic stage, lasting about 12 to 20 weeks, the blood was infective from time to time, and toxoplasms would thus be distributed to all tissues. Spleen, brain and lung were consistently infective, heart and liver often, thigh muscle and pancreas-omentum occasionally. This stage corresponds approximately to the duration of splenomegaly.

In the second stage, which continued until at least a year after inoculation, there was no evidence of circulating toxoplasms, and spleen and liver lost their

TABLE 1.

Distribution and persistence of Toxoplasma *in tissues of mice.*

Weeks after inoculation	Spleen index	Pseudocysts observed in brain	Splenomegaly in subinoculated mice								
			Brain	Lung	Heart	Thigh muscle	Spleen	Liver	Blood	Kidney	Pancreas omentum
1	72	−	−	+	−	−	++	−	−	++	−
2	105	−	++	++	−	−	++	++	++	−	−
3	140	N.E.	++	+	−	+	++	+++	+	−	−
4	143	−	++	++	−	−	+++	−	−	−	−
5	120	+	+++	+++	+++	−	+++	+	−	−	−
6	102	−	+++	+	−	−	+++	−	−	−	+
7	143	+	++	++	+++	−	+++	+++	+	−	+
8	133	+	+++	−	−	−	+	−	++	−	−
9	136	N.E.	+	+++	−	−	+	−	N.E.	−	−
10	133	N.E.	++	++	−	++	+++	+	N.E.	−	−
11	126	N.E.	+	−	+	+	+++	−	N.E.	−	++
12	99	+	++	−	−	−	+	−	−	−	−
14	114	−	+++	++	++	−	−	−	+	−	−
16	136	+	++	−	−	−	−	−	−	−	−
20	123	−	++	+++	+++	−	−	−	+	−	−
24	126	+	+	−	−	−	−	+	−	−	−
28	82	−	++	+++	+	+++	−	−	−	−	−
32	93	+	++	−	−	−	−	−	−	−	−
36	92	−	−	−	+++	+++	−	−	−	−	−
40	97	N.E.	+	++	−	−	−	−	−	−	−
44	90	N.E.	++	−	−	+	−	−	−	−	−
48	96	N.E.	+	+	+	+++	−	+	−	−	−
52	91	−	+	−	++	−	−	+	−	−	−
56		+									
60		+									
64		+									
68		−									
76		+									

+ : indicates that the mean spleen index (product of length and breadth in millimetres) of the three mice in the group exceeded 99, which is significantly greater than normal at the 0·05 level.

++ : mean spleen index >118.

+++ : mean spleen index >137.

N.E. : not examined.

infectivity. In almost every mouse, infectivity persisted in brain, and often also in heart, lung and thigh muscle.

After a year, the experiment became confused by the spontaneous development of leukaemia or lymphosarcoma in some of the inoculated mice, as well as in some of the uninoculated control mice kept in the same tins. Splenomegaly in these old mice, and in mice subinoculated from them, could no longer be accepted as presumptive evidence of toxoplasmic infection.

Results from subinoculation were supplemented with most mice by the search for *Toxoplasma* in sections of brain, lung and heart (stained with haematoxylin and eosin), and smears of brain (stained with Giemsa). Lung and heart were uniformly negative in this series. The brain results are included in Table 1. When toxoplasms were found, they established the infectivity of the

tissue, but, as they were very scanty in these earlier passages, failure to find them had little significance.

Behaviour in infant mice.—After intraperitoneal inoculation, these developed a very mild, inapparent infection. Splenomegaly was less frequently observed than in mice inoculated after weaning.

Behaviour of the Strain during Increase in Virulence for Mice.

A marked change in the behaviour of the organism occurred between the 64th and 84th passages, a period lasting from October, 1954, to September, 1955. One of the four mice of the 65th passage died nine days after inoculation, and two others, becoming ill, were sacrificed for passage on the 13th day. These showed inflammation of subcutaneous tissues, congestion of lymph nodes, and the presence of ascitic fluid. By the 84th passage, the mice invariably died. The survival time decreased; in the 72nd passage it was 12 to 16 days and, in the 84th, five days. Ascitic fluid became increasingly common, and typical toxoplasms were demonstrable in it. Extensive, red, oedematous consolidation of the lungs was a prominent feature in mice surviving more than seven days. Attempted cultures of the lungs on blood agar were negative.

Histological examination showed lesions that were, in general, more extensive and severe than those noted earlier, and toxoplasms were more readily demonstrable. A focal encephalitis, associated with either small pseudocysts or intracellular parasites, was present in the brain (Fig. 5). In the lungs, there

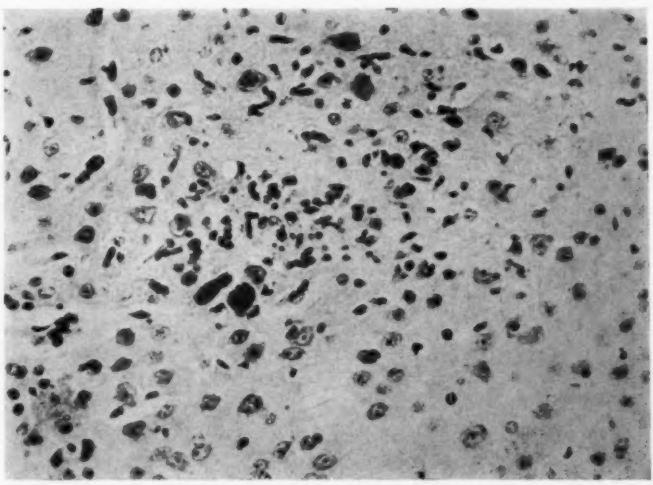

Fig. 5. Inflammatory focus, with small groups of *Toxoplasma*, in brain of mouse MQ12766 inoculated during the period of increase in virulence of the B316 strain. 16 days. H. & E. × 280.

were large areas of oedema and a scattered infiltration of cells (lymphocytes and mononuclears) in alveoli, in addition to the interstitial accumulations. The spleen in some cases showed areas of inflammation and necrosis in Malpighian bodies. Many foci of inflammation were seen in the liver.

Toxoplasms were most numerous in the fibrinous exudate on the peritoneal surfaces of abdominal organs and in the omentum and mesentery. They appeared usually as intracellular, spherical or crescentic bodies; large mononuclear cells might be packed with them. They were often seen also lying individually in the tissue spaces. They were present in smaller numbers in the substance of brain, liver, spleen and lung.

Behaviour of the Strain when Highly Virulent for Mice.

In the 85th, and later passages, intraperitoneally inoculated mice showed signs of illness at two days. The infection progressed rapidly, and always terminated in death, usually by the fifth day. Moribund mice were hunched and ruffled, and often the eyes were closed with exudate. Subcutaneous inflammation, slight enlargement of lymph nodes, and viscous ascitic fluid were regular autopsy findings. A thick, white exudate on the surfaces of the liver and spleen or in the mesentery was also common. Other abnormalities observed, in order of decreasing incidence, were general liver necrosis, gelatinous subcutaneous oedema, pleural fluid, and splenomegaly. Toxoplasms were very numerous in smears of ascitic fluid (Fig. 6) and in sections of peritoneal tissues, and histo-

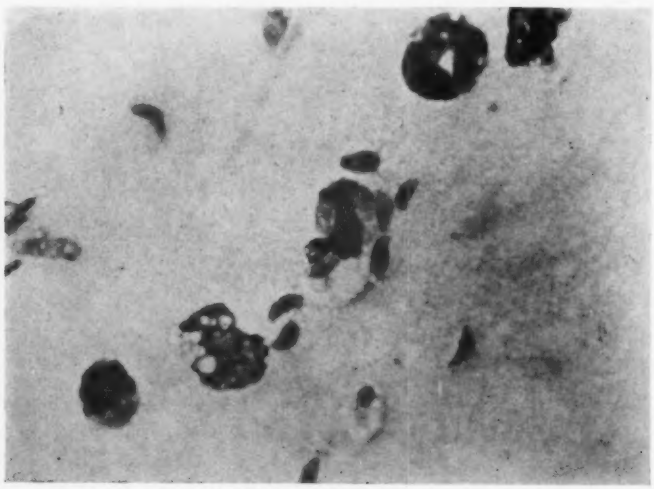

Fig. 6. *Toxoplasma* in the peritoneal fluid of a mouse inoculated with the virulent phase of the B316 strain. Giemsa. × 1100.

logical lesions in the organs were increasingly severe. At this stage, the inoculum for routine intraperitoneal passages was changed from liver and spleen to ascitic fluid.

Some mice of the 90th passage were inoculated intracerebrally, and all were moribund at five days. The skull was enlarged, and the brain firmly adherent to the dura. Numerous free round organisms were present in brain sections.

Behaviour in infant mice.—As was found with the avirulent phase, infant mice appeared to be more resistant than weaned mice. A litter of six one-day-old mice inoculated intraperitoneally at the 90th passage survived from 9 to 22 days. Three weaned mice inoculated with the same material were moribund in five days.

Behaviour in guinea-pigs.—Infective material of the 82nd mouse passage was inoculated into four guinea-pigs, and five serial intraperitoneal transfers made. Liver and spleen were used each time, but a small quantity of peritoneal fluid was included in the first two transfers, and lung in the fourth and fifth. Twelve guinea-pigs were used. In the first passage, each guinea-pig showed a febrile response of three or four days duration, commencing two or three days after inoculation. One also showed a second febrile period between 9 and 11 days. In the five following passages, seven of eight guinea-pigs showed a febrile period of one to four days, commencing four to six days after inoculation. The febrile response showed some variation, as is illustrated in Figs. 7 and 8.

Splenomegaly occurred in five of seven guinea-pigs examined within 10 days of inoculation, but not in four examined between 22 and 40 days. No other lesions were observed.

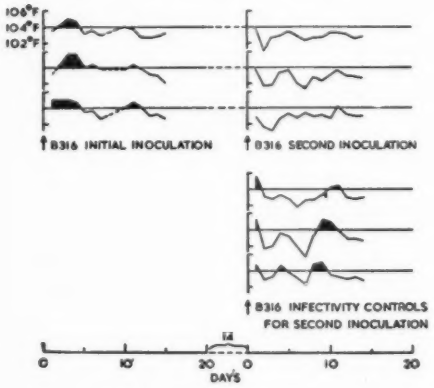

Fig. 7. Guinea-pig temperature charts, showing response to the B316 strain, and resistance to reinoculation.

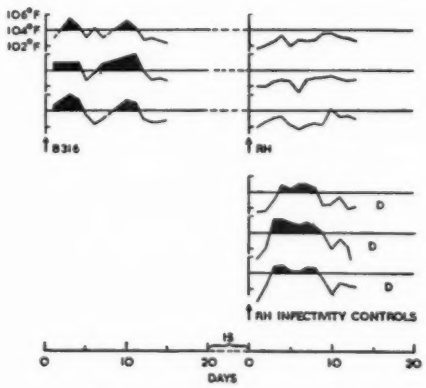

Fig. 8. Guinea-pig temperature charts, showing resistance of guinea-pigs infected with the B316 strain to subsequent inoculation with the RH strain.

At each guinea-pig transfer, a group of four to six mice was inoculated as well. The mortality in these mice was almost 100 p.c. Organisms were demonstrated, usually in small numbers, in the ascitic fluids of mice inoculated with tissues of guinea-pigs of the first, second, third and fifth passages. The guinea-pigs of the first passage were tested for infectivity up to 32 days by inoculation of mice with positive results.

Behaviour in rats.—Two rats were inoculated intraperitoneally with infective mouse peritoneal fluid. They showed no signs of illness, and were autopsied 15 months later. Organisms were not recovered in mice from brain, heart and spleen of the rats.

Two other young rats were similarly inoculated, and had shown no illness when they were autopsied at 21 days. The serum of each had a high complement fixing antibody titre (Table 2), but *Toxoplasma* was not recovered by three passages in mice from a pool of liver, spleen and brain of the rats.

TABLE 2.

Reciprocal complement fixation titres in rats.

Rat No.	Inoculum	Antigen	
		B316	RH
3	B316 strain	256	512
4	B316 strain	256	256
9	RH strain	32	64
10	RH strain	512	512
7	Normal mouse tissues	0	0
8	Normal mouse tissues	0	0

Intracerebral inoculation of four rats with mouse peritoneal fluid produced no apparent illness, but *Toxoplasma* was demonstrated in mice inoculated with brain of rats killed at 45 and 87 days.

The B316 strain thus appeared to be of low virulence for rats.

Behaviour in rabbits.—Two adult rabbits were inoculated intraperitoneally with 0·4 ml. of mouse ascitic fluid containing numerous toxoplasms. Both became febrile within one or two days, and remained so for six or seven days, before recovering. Twenty-six days after inoculation, they were sacrificed, and the brain of each was inoculated into mice. However, the mice remained well, and *Toxoplasma* was not recovered from the rabbits.

It was concluded that the B316 strain was not of high virulence for rabbits.

Behaviour in chick embryos.—Inoculation of infected mouse ascitic fluid into the allantoic cavity of 11-day eggs resulted in fatal infections in 6-8 days. Numerous pocks about 0·5-2·0 mm. diameter were present on the allantoic membrane.

Identification.

In 1941, Sabin reported the isolation, from a boy of six, of the RH strain of *Toxoplasma*. This has been maintained, and generally accepted as a strain of *T. gondii*. It is now widely used as a reference strain in the immunological study of newly isolated organisms.

A study of the relationship of the B316 strain to the RH strain was made by means of immunity tests and cross complement fixation tests.

Three tests were made in guinea-pigs, to determine if infection with the B316 strain produced immunity to the homologous strain and to the RH strain. In the first immunity test (Fig. 7), three guinea-pigs were infected with the B316 strain. Thirty-four days later, they were reinoculated with the B316 strain, and showed no significant febrile reaction, while three other previously uninoculated guinea-pigs of similar size, which received equal amounts of the same inoculum, each showed two days of fever.

In the second immunity test (Fig. 8), three guinea-pigs were infected with B316 strain, and thirty-five days later were inoculated with the RH strain. No significant febrile reactions occurred, and all three guinea-pigs remained well up to 28 days. Equal amounts of the same RH inoculum produced a marked febrile response in each of three previously uninoculated guinea-pigs of similar size, and they died at 15, 16 and 17 days.

Sera, obtained by heart puncture, of the twelve guinea-pigs used in the first two tests, were found to possess no complement fixing antibodies before inoculation.

In the third test, the three guinea-pigs of the first test, which had already been twice inoculated with B316 strain, were inoculated with a larger dose of RH strain. No significant febrile responses occurred, and all three remained well up to 28 days, when they were discarded, while the three control guinea-pigs receiving a similar dose of RH strain showed brief febrile periods and died within five days.

Complement fixation tests were performed by the method of Lennette *et al.* (1949). B316 and RH antigens were prepared according to Warren and Russ (1948), with the additional centrifugation recommended by Sabin (1949). Each strain was inoculated into two rats, B316 intraperitoneally and RH subcutaneously. Also, two rats were inoculated intraperitoneally with normal mouse liver and spleen. Sera were collected at 21 days. These tests indicated that a close serological relationship existed between the two strains (Table 2).

The serum of Bandicoot 316 was also tested for complement fixing antibodies against both the B316 and RH antigen, and showed titres of 1/128 and 1/64, respectively.

Although the B316 and RH strains were immunologically similar, there were differences in their behaviour in laboratory animals. Whereas the B316 strain was lethal for mice only, the RH strain was rapidly lethal for mice, rats and guinea-pigs, and Sabin (1941) reported that it was also lethal for rabbits.

DISCUSSION.

In Australia, *Toxoplasma* has been reported in both animals and humans, mainly on histological studies. Infection in laboratory rabbits, a sheep, a cat and three dogs was recorded by Wickham and Carne (1950). Seddon (1952) mentions infection in an Adelaide rabbit colony, and in a marsupial native cat, *Dasyurus quoll* (Zimmermann), observed by Carne. Organisms resembling *Toxoplasma* were reported to be present in sparrows (*Passer domesticus* Linné) examined by Lawrence (1946), but more information would be necessary for identification. At least four fatal human cases have been recorded in Australia, one of which occurred in Brisbane.

There have been previous reports of *Toxoplasma* in other marsupials besides the native cat. Hackel *et al.* (1953) reported *Toxoplasma* in a kangaroo, *Macropus rufus* (Desmarest), in Saint Louis Zoo. Two wallabies, *Macropus bennetti*[1] in the Philadelphia Zoological Garden were found to be infected by Ratcliffe and Worth (1951). Two wombats, *Phascolomys mitchelli*[2], were reported to be infected in London (Coutelen, 1932). Although these last five infections were detected in other countries, they show the susceptibility of some marsupials to *Toxoplasma*. The bandicoot, *T. obesulus*, is thus the fifth marsupial species to be found naturally infected.

The B316 strain is remarkable for its extraordinary low virulence for mice, which continued through many passages for 44 months from its first isolation. Not until the 65th passage did it cause the death of a mouse. With further passage, the virulence steadily increased, until the mortality with the routine transfer technique became 100 p.c. The increase in virulence of an infective agent with repeated animal passage is, of course, a widely observed phenomenon. Jacobs and Melton (1954) have described the increase in virulence with mouse passage of their strain 113 of *Toxoplasma*. Its history began with a mouse inoculated with 45 adult *Rhipicephalus sanguineus*, which had fed as nymphs on a rabbit infected with the RH strain. The first mortality appeared with the 14th mouse passage (there were eight intervening passages in chick embryos). Thereafter, the virulence continued to increase. Passage in chick embryos did not increase virulence for mice.

There is a close analogy between the course of infection in mice inoculated with the B316 strain in its phase of low virulence and the course of toxoplasmosis in man. In both, the parasitaemic stage is followed by the localization of toxoplasms in certain organs, particularly the brain. In the human adult, infection is usually inapparent, but may become manifest by foetal damage, if the parasitaemic stage coincides with pregnancy. A feature of the parasitaemic stage in the mice was a transient interstitial pneumonitis. A similar lesion in fatal human cases has been reported by several authors, including Pinkerton and

[1] Syn. of *Wallabia rufogrisea* (Desmarest).
[2] Syn. of *Vombatus hirsutus* (Perry).

Henderson (1941), Frenkel and Friedlander (1951) and O'Reilly (1954) in a case occurring in Brisbane. It seems probable that some cases of transient, non-fatal human pneumonitis could be of toxoplasmic origin.

SUMMARY.

A strain of *Toxoplasma gondii* (B316) was isolated from a new host, a naturally infected bandicoot, *Thylacis obesulus*, in Brisbane. Pseudocysts were present in the brain of the bandicoot, and its serum had complement fixing antibody titres of 1/128 and 1/64 to B316 and RH strain antigens respectively.

A close immunological relationship between the B316 and RH strains was shown by cross complement fixation tests, and by the absence of reaction of guinea-pigs previously infected with B316 when challenged by RH.

The B316 strain was of very low virulence for mice when first isolated, the first 64 intraperitoneal passages producing only an inapparent infection. The virulence for mice then increased, and from the 85th passage the mortality rate was 100 p.c. with a usual survival time of five days. The strain was then lethal for chick embryos also, but not for guinea-pigs, rabbits or rats.

There is a resemblance between the usual course of toxoplasmosis in the human adult, and that in mice infected with B316 in its earlier phase of low virulence. The occurrence in the mice of a transient focal interstitial pneumonitis suggests that a simlar condition in man might sometimes be of toxoplasmic origin.

Acknowledgments. We are indebted to Miss Yvonne J. Berglin and Mr. J. G. Carley for help with the mouse and egg studies, to Mr. P. B. McGovern for statistical advice, to Dr. J. K. Frenkel of the University of Kansas for comments on the parasite as seen in stained slides, and to Dr. E. L. French of the Walter and Eliza Hall Institute, Melbourne, for the RH strain.

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TOXOPLASMA IN QUEENSLAND.

II. NATURAL INFECTIONS IN BANDICOOTS AND RATS.

by J. H. POPE, V. A. BICKS, AND I. COOK

(From the Queensland Institute of Medical Research, Brisbane).

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In the preceding paper (Pope *et al.*, 1957), the isolation, identification and characteristics of the B316 strain of *Toxoplasma* were described. These findings naturally led to a more complete investigation of the possible rôle of the bandicoot as a reservoir of *Toxoplasma*. Rats from the same habitats were also studied for comparison.

METHODS AND MATERIALS.

Animals were trapped alive at night between August, 1955, and February, 1956, the species captured being *Thylacis obesulus* (Shaw and Nodder), *Perameles nasuta* Geoffroy, *Rattus assimilis* (Gould), *R. norvegicus* (Berkenhaut), and *R. rattus* (Linné). Usually they were not held more than a few days; but five bandicoots were held in separate cages in a special animal house for a month, one for 5½ months, and one for seven months, before autopsy.

Animals were killed with town gas, weighed, and dissected aseptically. The usual procedure with bandicoots was to prepare two inocula. One was made from pooled liver, spleen, kidney, lung and heart, and 0·5 ml. of the suspension was inoculated intraperitoneally into four mice, while the other was made from brain, and 0·03 ml. was inoculated intracerebrally into four mice. Sometimes, bandicoot blood for mouse inoculation was taken from the exposed jugular vein, before the abdomen was dissected. Blood was also taken from the heart for serum and for smears. The spleen was weighed. Portions of the liver, spleen, kidney, lung, heart and brain were fixed in phosphate-buffered 10 p.c. formalin, and, after paraffin embedding, sections were cut at 5-6 μ and stained with haematoxylin and eosin.

Any ectoparasites present were collected alive, and, after identification, or preservation of some for identification, a suspension was prepared. This was inoculated into two to four mice intraperitoneally, or intracerebrally, or both. Occasionally, suspensions of endoparasites were inoculated into mice.

With *R. assimilis*, the procedure was similar to that used with the bandicoots, except that the spleen size was measured in sq. mm. (length × breadth).

In dealing with *R. rattus* and *R. norvegicus*, only one inoculum was prepared, from pooled liver, spleen, kidney, lung, heart and brain, and it was inoculated intraperitoneally. Here again, the spleen sizes were measured, and a few weights were also recorded.

All tissues, as well as ecto- and endoparasites, were ground with alundum, and suspended in saline. A few minutes were allowed for settling, and the supernate was used as inoculum. With the ecto- and endoparasites, penicillin and streptomycin were added in concentrations of approximately 400 and 1500 units, respectively, per ml. of inoculum, which was then held in the refrigerator for 30 minutes before inoculation.

The strain of mice used in this work was that previously described (Derrick *et al.*, 1954). No evidence of enzootic infection with *Toxoplasma* has been found in the colony. Three passages at 14-day intervals were made in mice, before dismissing an attempt at isolation as negative. Liver and spleen were used for intraperitoneal mouse passages, and brain for intracerebral passages. If signs of *Toxoplasma* infection were present in the mice at autopsy, Giemsa-stained brain contact smears were prepared, usually from the intracerebrally inoculated mice. When isolation of *Toxoplasma* was confirmed by finding pseudocysts in the mouse brains, fortnightly passages were not continued, but the mice were allowed to remain as carriers.

Toxoplasma antigen was prepared from chorio-allantoic membranes of infected embryonated eggs, and a control antigen from an extract of normal membranes of the same age. The additional centrifugation to remove nonspecific components was carried out in a Spinco Model L Ultracentrifuge at 13,000 r.p.m. for one hour in the cold.

In performing the complement fixation tests, it was found convenient to use complement preserved with the boric acid-sodium acetate mixture of Campell and Turner (1953), and sheep blood taken into the glucose-citrate-merthiolate solution of Stubbs (1953). New batches of antigen were titrated in the "checkerboard fashion" against the serum of a guinea-pig, which had been inoculated with the B316 strain and bled after an interval of four weeks.

Isolations.

Using the methods described, 23 strains of *Toxoplasma* were isolated from 83 bandicoots and rats examined (Table 1). *Toxoplasma* was isolated from approximately 40 p.c. of bandicoots. The incidence in rats was lower. First signs of infection (splenomegaly, and sometimes macroscopic focal lung lesions) occurred most commonly in mice of the first passage, rarely in mice of the second passage, and never in mice of the third passage.

TABLE 1.

Isolation of Toxoplasma *from bandicoots and rats.*

Animal	Locality	No. inoculated into mice	No. isolations
T. obesulus	Brisbane	38	16
	Mt. Glorious	2	0
	North Queensland	3	1
		43	17
P. nasuta	Mt. Glorious	2	1
	North Queensland	4	2
		6	3
R. assimilis	Mt. Glorious	12	2
R. norvegicus	Brisbane	15	1
R. rattus	Brisbane	7	0

Host Range and Virulence.

These strains behaved like the avirulent phase of the B316 strain described in the previous paper, in that intraperitoneal inoculation of mice produced no apparent symptoms. However, intracerebral inoculation usually produced a slight illness, indicated by a ruffled, hunched appearance of the mice. In one instance, when serial intracerebral passages were made from the brain of *T. obesulus* No. 446, mice became more severely affected, and some died, with numerous free organisms and pseudocysts in brain smears, after four intracerebral passages. A high mortality was maintained in subsequent passages, and the survival time was about 5-7 days in the 10th passage.

Six strains tested (three from *T. obesulus*, one from *P. nasuta*, one from *R. assimilis*, and one from *R. norvegicus*) produced a febrile reaction in 11 of 17 guinea-pigs inoculated. Four of these strains (three from *T. obesulus*, and one from *R. assimilis*) were also tested for pathogenicity in rats by attempting to recover the organism in mice after 21-25 days. Positive results were obtained with two strains from *T. obesulus*. No deaths occurred in any of the guinea-pigs or rats. Thus, the strains tested were only mildly pathogenic for these hosts.

Identification of Strains.

Four strains, representative of those isolated from *T. obesulus*, *P. nasuta*, *R. assimilis*, and *R. norvegicus*, were studied by means of guinea-pig immunity tests. One month after inoculation with the test strain, in the form of infected mouse tissues, two guinea-pigs were challenged with a lethal dose of peritoneal fluid from a mouse infected with RH strain. None of the previously inoculated guinea-pigs died from the RH challenge, and, except with the strain isolated from *R. norvegicus*, none showed a febrile response. Both control animals became febrile, and died in five to twelve days.

The positive results in complement fixation tests on bandicoot and rat sera were further evidence that the isolated organisms were *Toxoplasma gondii*. Both B316 and RH antigens were used. The results also confirm that the strains originated from the wild animals and not from the mouse stock.

Serology of the Wild Animals.

The results of complement fixation tests with the B316 antigen on the sera of the wild animals are shown in Table 2. There was good correlation between the presence of complement fixing antibodies and the isolation of *Toxoplasma*, so the complement fixation test appears to be a reliable method of detecting chronic infections in these animals.

Two of 17 bandicoots from which toxoplasms were isolated gave negative complement fixation tests with the B316 antigen. One of these, *T. obesulus* No. 464, was in the parasitaemic stage, and it may have been too early in the infection for antibody formation. The serum of the other bandicoot, *T.*

TABLE 2.

Correlation between complement fixation test and animal inoculation.

Animal	Isolation result	No.	No. anti-complementary	Complement fixing antibody titre						
				0	1/8	1/16	1/32	1/64	1/128	1/256
T. obesulus	+	16		2		1	6	7		
	−	21	2	18	1					
P. nasuta	+	1								1
R. norvegicus	+	1	1							
	−	14	2	12						
R. assimilis	+	2							2	
	−	10	2	7		1				
R. rattus	−	7	1	6						

obesulus No. 441, had a titre of 1/64 with the RH antigen. Otherwise, the results obtained with the B316 and RH antigens agreed.

The sera of both animals (*T. obesulus* and *R. assimilis*), which were negative by mouse inoculation and positive by complement fixation test, had low titres (1/8). Both might have been infected and subsequently eliminated the organism, or the results might be due to incomplete specificity of the test at that titre.

No evidence was found of cross reaction between *Toxoplasma* and other parasites present in the animals. Thus, in those from which *Toxoplasma* was not isolated, *Theileria* sp. was observed in nine *T. obesulus*, *Sarcocystis* sp. in one *R. assimilis* and one *R. rattus*, *Hepatozoon muris* (Balfour) in six *R. assimilis* and ten *R. norvegicus*, *Trypanosoma lewisi* (Kent) in three *R. assimilis* and two *R. norvegicus*, *Spirillum minus* Carter in five *R. norvegicus*, and *Haemobartonella muris* (Mayer) in nine *R. norvegicus*. The serum of only one of these animals, a *T. obesulus* with a *Theileria* infection, fixed complement; the titre was 1/8.

Pathological Examination of Wild Animals.

Bandicoots.—All of the bandicoots appeared well, prior to autopsy. Splenomegaly, small splenic cysts, small lung nodules, liver enlargement, and enlarged lymph nodes were the only abnormalities seen at autopsy. However, none of these showed any consistent relationship to infection with *Toxoplasma*. Spleen sizes (expressed as percentage of body weight) averaged 1·1 for 19 uninfected bandicoots, and 1·0 for 14 which were infected. Lung-worms were usually associated with the spots on the lungs.

Tissue sections were studied of 42 bandicoots, of which 17 were infected. Mild encephalitis was observed in 16 of the 17 infected bandicoots, but in none of the uninfected. Perivascular cuffing was present in each of the 16.

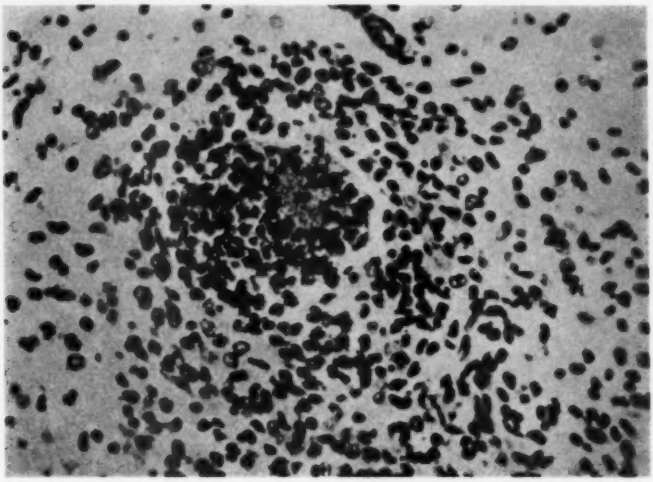

Fig. 1. Ruptured pseudocyst with severe inflammatory reaction in brain of *P. nasuta* No. 403. H. & E. × 465.

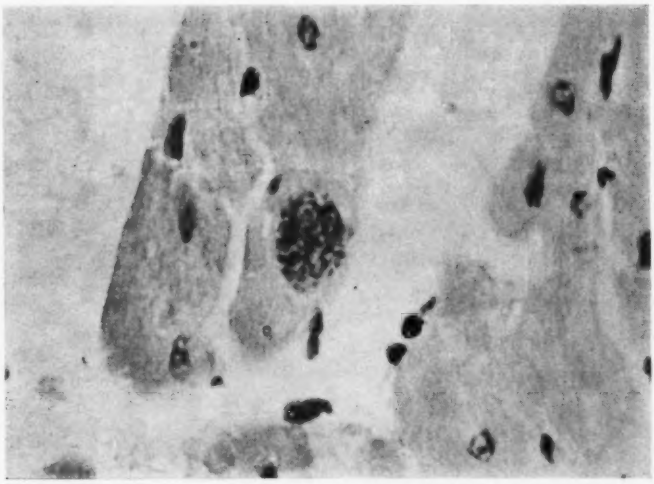

Fig. 2. Transverse section of an intracellular pseudocyst in heart of *T. obesulus* No. 441. H. & E. × 780.

Pseudocysts, up to 72 μ diameter, were seen in 13. Sometimes they were plentiful, but in other cases considerable searching was necessary to demonstrate them. Intact pseudocysts were free from any reaction, but a large, apparently ruptured pseudocyst in the brain of *P. nasuta* No. 403 was associated with a severe inflammatory reaction (Fig. 1). Small inflammatory foci were seen in the brains of eight animals.

After the brain, the tissue most commonly affected was heart. Foci of myocarditis (5), pseudocysts (5), or both (2), were seen in 12 of the 17 infected bandicoots. Only in one uninfected *T. obesulus* was a small, possibly inflammatory, group of cells seen. The myocarditis was usually mild and localised. Organisms were not found associated with it. No inflammatory reaction occurred about the pseudocysts. In one bandicoot, *T. obesulus* No. 441, transverse sections of small pseudocysts within the intact muscle fibre were seen, clearly showing the intracellular location of the pseudocysts in the heart (Fig. 2). In another, *T. obesulus* No. 467, pseudocysts were found in the heart, although they could not be found in the brain.

A small group of toxoplasms and mild interstitial pneumonitis were observed in the lung of *T. obesulus* No. 464, which had parasitaemia. Thickening of the alveolar septa, was present in some other infected animals, and may have been due to infection with *Toxoplasma*. However, foci of inflammation apparently not due to toxoplasms were observed in the lungs of many of the bandicoots of this series, both infected and uninfected.

No abnormalities attributable to *Toxoplasma* were found in liver, spleen or kidney.

R. norvegicus. The *R. norvegicus* No. 686, from which *Toxoplasma* was isolated, was a large male weighing 455 gm. Its spleen was no larger than those of other rats of this species of comparable size in the series. Small haemorrhagic spots and cream nodules were present on the lung surfaces. Meischer's tubules, in which *Sarcocystis* spores were demonstrated, were present in the diaphragm and the abdominal wall.

In the brain sections, slight cuffing of minor blood vessels was observed, and also a small inflammatory lesion. Severe myocarditis was present, but no organisms were seen. An exudate containing many polymorphonuclear cells was present in the bronchioles and also in the lung tissue, in some places causing severe distension of alveoli. In some parts of the lung there were areas of thickening of the alveolar walls, with numerous polymorphonuclear leucocytes. It seems likely that a lung infection was superimposed on the *Toxoplasma* infection.

R. assimilis. VA 44 was a pregnant female. The spleen index was 247, larger than that of any of the uninfected rats of this species examined. One eye had a white opacity over the pupil. Lung-worms, *Angiostrongylus cantonensis* (Chen.), were present, and ascarid larvae, *Amplicaecum* sp., were seen in the liver. Histologically, pseudocysts, mild meningitis, perivascular

cuffing, and a small inflammatory lesion, were observed in the brain. Other tissues appeared normal.

VA 55 was a male, weighing 183 gm. Splenomegaly was present (index 405). A right axillary lymph node was enlarged (8 mm. diameter), and there was a slight scab in the skin over it. Ascarid larvae were present in the liver. Two small inflammatory nodules were the only abnormalities observed in brain sections. Mild myocarditis was present in the form of small scattered foci. Small, spherical, strongly basophilic bodies of variable size, probably chromatinic debris, were present in macrophages of a lymph node and of the spleen. Polymorphonuclear cells were also numerous in the lymph node, and there was a small area of degeneration. Toxoplasms were not seen in the lymph node, so there was no evidence to suggest that infection with *Toxoplasma* occurred through the skin wound. Lung and liver appeared normal.

Distribution of Toxoplasma *in Bandicoots.*

Further information on the distribution of the organisms in naturally infected bandicoots was obtained by mouse inoculation studies.

Sixteen tissues or body fluids from *T. obesulus* No. 447 were individually tested for infectivity by mouse inoculation. Initial inoculations were by the intraperitoneal route, and at least one blind passage was made. When splenomegaly or focal pneumonitis developed in mice, intracerebral passages were begun, and brain smears were searched for pseudocysts. In this way it was shown that brain, heart, inguinal lymph nodes, and a pool of pancreatic and omental tissue were infective. *Toxoplasma* was not isolated from blood, spleen, liver, kidney, lung, skeletal muscle, testis, eye, bone marrow, lumbar spinal cord, skin and urine.

With sixteen other infected bandicoots, all brains were infective for mice, as were also all thirteen pools of organs tested. Blood from the jugular was positive in only one of five bandicoots tested (*T. obesulus* No. 464), and urine was negative on two occasions.

Three chronically infected bandicoots carried suckling young, which were also tested for infectivity by inoculation of their brains into mice, but the results were negative.

The consistency of infectivity of bandicoot brain was confirmed by the histological study, which also suggested that the heart was contributing significantly to the infectivity of the pools.

Examination of Ecto- and Endoparasites by Mouse Inoculation.

The inoculated parasites are listed in Table 3. They included several batches from uninfected animals, as it was not known at autopsy whether the animal was infected or not. The most significant are nine batches of ectoparasites from infected bandicoots, two being from an animal with proven parasitaemia. All were negative, and no evidence was obtained of participation by either ecto- or endoparasites in the transmission of *Toxoplasma.*

TABLE 3.

Examination of parasites by mouse inoculation.

Parasite	No. of batches	No. of batches from animals with *Toxoplasma* infection	Host
FLEAS			
Stephanocircus sp.	11	2, chronic	*T. obesulus*
Pygiopsylla sp.		1, parasitaemic	
TICKS			
Ixodes holocyclus Neumann	12	2, chronic	*T. obesulus*
Ixodes holocyclus Neumann	1		*P. nasuta*
Ixodes tasmani Neumann	9	2, chronic	*T. obesulus*
		1, parasitaemic	
MITES			
Peramelaelaps bandicoota Womersley	1		*T. obesulus*
Mesolaelaps anomalus Hirst	1		*T. obesulus*
LICE			
Boopia sp.	1	1, chronic	*T. obesulus*
PANCREATIC FLUKES			
Zonorchis sp.	2	2, chronic	*T. obesulus*
ACANTHOCEPHALA			
Moniliformis semoni (v. Linstow)	1		*T. obesulus*
LUNG WORMS			
Marsupostrongylus bronchialus Mackerras and Sandars	5	3, chronic	*T. obesulus*
STOMACH WORMS			
Trichostrongylidae (undescribed sp.)	1	1, chronic	*T. obesulus*
INTESTINAL NEMATODES			
Echinonema cinctum (v. Linstow)	3	2, chronic	*T. obesulus*
LIVER WORMS			
Amplicaecum sp. (larvae)	1		*R. assimilis*

Distribution of Infected Bandicoots and Rats.

Isolations of *Toxoplasma* were made from bandicoots (*T. obesulus*) and a rat (*R. norvegicus*) in the western suburbs of Brisbane, from a bandicoot (*P. nasuta*) and rats (*R. assimilis*) at Mt. Glorious, a small rural settlement about 30 miles north-west of Brisbane, and from bandicoots (*T. obesulus* and *P. nasuta*) in the Innisfail district, north Queensland (Table 1).

DISCUSSION.

The difference in virulence of various strains of *Toxoplasma* has been recently reviewed by Lainson (1955). In the present work, the initial virulence for mice has been consistently low in all of the strains from both bandicoots and

rats. In contrast, the strains isolated from *R. norvegicus* by Perrin *et al.* (1943) and by Eyles (1952) were lethal to mice.

The healthy appearance of the infected bandicoots may mean that the strains are relatively avirulent for these species, but experimental infections in them have not yet been studied. Even though these strains were not lethal for the few species of animals examined, it cannot be assumed that their behaviour in man would be equally mild. At least four deaths from toxoplasmosis have been recorded in Australia.

The main finding in this work is that bandicoots are important hosts of *Toxoplasma* in Queensland. Evidence for this conclusion is the high incidence of infection found during the period of study, and the presence of infected bandicoots in three different areas of the State. The first isolation (B316 strain) was made 4½ years earlier. The presence of *Toxoplasma* in rats is also of epidemiological interest, in spite of the lower incidence of infection.

Bandicoots are widespread and numerous in the coastal area of Queensland. They are commonly present in suburban gardens, but usually do not enter houses, and so may not have very direct contact with humans. One possible way of transfer of the organisms from bandicoots to man would be by rats. However, it is more likely that dogs and cats, which are free to roam at night in the same areas as the bandicoots, could occasionally become infected. Subsequently, during the acute stage of the infection, these pets would have chances of close contact with their owners. Dogs are known to be susceptible to infection with *Toxoplasma*, and numerous natural infections have been recorded. Thus, although the bandicoot is rarely seen by most people, only a link, such as the one suggested, is needed to increase considerably the risk of human infection.

Most reports of *Toxoplasma* in animals describe either sporadic cases, certain limited epizootics, such as in dog kennels, or enzootic infections in rabbit or guinea-pig colonies. Larger, free-living reservoirs, such as the hares in Denmark (Christiansen and Siim, 1951) and Sweden (Borg, 1953), *R. norvegicus* in the United States (Perrin *et al.*, 1943; Eyles, 1952), and the bandicoots in Queensland, are less commonly described. They are possibly more significant, however, because the sporadic cases in man perhaps originate from them. Once the reservoirs become well-known, and transmission is understood, the origin of sporadic cases will become less baffling than at present.

SUMMARY.

Two new hosts of *Toxoplasma* are recorded; the bandicoot, *Perameles nasuta*, and the native rat, *Rattus assimilis*. A natural infection in *R. norvegicus* is also recorded.

Toxoplasma was isolated from three centres in Queensland—Brisbane, Mt. Glorious and Innisfail.

The incidence in bandicoots was about 40 p.c., indicating that they are a substantial reservoir of infection.

No gross abnormalities were seen at autopsy, but encephalitis, myocarditis, and pseudocysts were demonstrated histologically.

The organisms were isolated in mice in each instance. The virulence for mice was generally very low, with slight variation with some strains.

The strains tested were found to be pathogenic for guinea-pigs, but again the virulence was low.

Representative strains from each host species were shown to be related to the RH strain of *Toxoplasma* by guinea-pig immunity tests.

Complement fixing antibodies to B316 *Toxoplasma* antigen were present in almost all of the infected animals, but rarely in those that were uninfected.

Attempts to isolate *Toxoplasma* from various parasites of the bandicoots gave negative results.

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THE ACTION OF SALICYLATE, DINITROPHENOL AND CARDIAC GLYCOSIDES ON THE ISOLATED TOAD HEART

by WINIFRED G. NAYLER[1]

(From the Baker Medical Research Institute, Prahran, Melbourne).

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Up to the present, studies on the importance of oxidative phosphorylation reactions in the cardiac response to the glycosides have not yielded conclusive results. Thus Ellis (1953), using the hypodynamic frog's heart and Wollenberger and Karsh (1952) using the depressed Langendorff heart reported that 2:4-dinitrophenol (D.N.P.) inhibited the positive inotropic action of the digitalis drugs, whilst Taeschler *et al.* (1953) and Rothlin *et al.* (1955) were able to display a positive inotropic action of lanatoside-C on the dog heart-lung preparation in which mild failure had been induced by D.N.P.

These workers have all used D.N.P. to limit the rate of oxidative synthesis of high energy phosphate compounds — this agent dissociates oxidation and phosphorylation (Loomis and Lipman, 1948). Recently, Smith and Jeffrey using isolated rat diaphragm (1956) and Sproull (1954) using mouse liver slice have demonstrated that the salicylates, like D.N.P., reduce the production of high energy phosphate-bond compounds. This may be due to interference with oxidative phosphorylation or to a changed rate of creatine phosphate turnover by Lohmann's reaction.

The present investigation was carried out to compare the action of D.N.P. and sodium salicylate on the isolated perfused toad heart, and to determine what effect, if any, these two inhibitors had upon the response of the heart to glycosides.

Method.

The modified Warburg type respiratory apparatus previously described (Nayler and McKelvie, 1956) was used throughout these experiments for the simultaneous observations of oxygen uptake, stroke volume, beat rate and maximum aortic pressure of the isolated spontaneously beating toad heart. Observations were made at $25{\cdot}0 \pm 0{\cdot}05°$ C., using unselected non-hibernating male and female toads of the species *Bufo marinus*. The standard perfusion fluid contained 3 gm. glucose/litre as the sole exogenous substrate.

Since only changes in oxidative metabolism produced by the action of the inhibitors or the glycosides in the presence of these inhibitors were being studied, the usual carbon dioxide measurements and efficiency calculations were not made in this series.

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[1] The expenses of this investigation were defrayed by a grant from the Life Insurance Medical Research Fund of Australia and New Zealand.

The experimental procedure was essentially the same as that used in previous studies using this apparatus. All hearts were initially perfused for forty minutes in standard Ringer solution or Ringer containing the appropriate inhibitors, during which time oxygenation and temperature equilibration were complete. This was followed by a standard one hour's perfusion – during which observations were made at fifteen-minute intervals – before the addition of the drugs to the perfusate. Subsequently, observations were continued for three hours in the control, salicylate and normal glycoside series, and for two hours in the D.N.P. series. All solutions of drugs were made in the standard Ringer.

Salicylate-Dinitrophenol Series.

In six experiments using the standard perfusate throughout, observations of oxygen uptake, stroke volume, beat rate and maximum aortic pressure were measured at fifteen-minute intervals during four hours continuous perfusion. Similarly in six experiments sodium salicylate (5 mM final concentration) and in four experiments D.N.P. ($1{\cdot}09 \times 10^{-5}$M final concentration) was initially added to the perfusate and readings taken throughout the four hours.

The action of sodium salicylate when added to the heart in the above concentration after the control one hour's perfusion in standard Ringer was recorded for three hours in six experiments; the effect of D.N.P., when similarly added in the above concentration after the initial one hour's perfusion, was recorded in four preparations. In this latter series observations subsequent to the addition of the inhibitor to the heart were restricted to two hours, since prolonged perfusion in D.N.P. Ringer produced a marked change in the rate of oxygen uptake – see Fig. 2.

Inhibitors plus Glycosides Series.

The effects of strophanthin-G[2] (1×10^{-5} final concentration) and lanatoside-C[3] (2×10^{-5} final concentration) when added to hearts previously perfused for one hour and forty minutes with Ringer containing sodium salicylate (5 mM final concentration) were recorded; in both cases six experiments were performed and observations made at fifteen-minute intervals one hour before and three hours subsequent to the addition of the glycosides to the perfusate.

In a further series strophanthin-G plus sodium salicylate, and lanatoside-C plus sodium salicylate, were added to the heart in the above concentrations after an initial one hour and forty minutes perfusion in the standard perfusate. In each case six experiments were performed and observations made as above throughout four hours.

D.N.P. and Strophanthin-G.

The action of strophanthin-G (1×10^{-5}) when added to hearts previously perfused for one hour and forty minutes with perfusate containing D.N.P. ($1{\cdot}09 \times 10^{-5}$M) was recorded in four preparations. In a similar series strophanthin-G plus D.N.P. were added together (in the above concentrations) after the initial perfusion in standard perfusate.

These results have been compared with those reported from previous studies in which strophanthin-G (Nayler, 1956) and lanatoside-C (Nayler, 1957) were added in the above concentrations to similar preparations perfused in standard Ringer.

RESULTS.

The results obtained throughout this study are shown in Figs. 1-5. In each case only the mean results are displayed.

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[2] Strophanthin-G: as Ouabain (Arnaud): Laboratoire Nativelle product.
[3] Lanatoside-C: Cedilanide Sandoz Ltd. product.

When perfused throughout four hours with the standard perfusate a relatively constant beat rate and maximum aortic pressure were recorded, together with a gradual decline in cardiac output (expressed as drops per beat) and a slow, steady rise in oxygen utilization during the last two hours of perfusion—see Fig. 1 A-D.

In those preparations in which salicylate (5 mM final concentration) was present throughout the entire perfusion the beat rate, stroke volume and maximum aortic pressure records followed a pattern similar to that in the normal

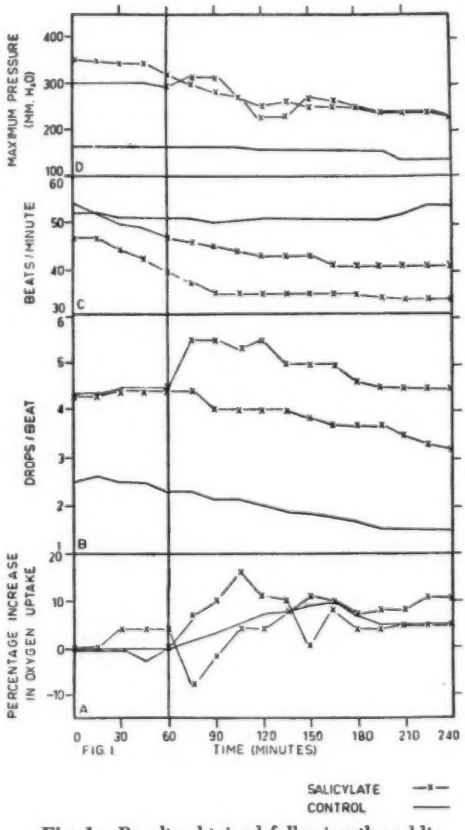

Fig. 1. Results obtained following the addition of sodium salicylate (5 mM final conc.) to the preparation at the beginning and after one hour's perfusion in standard Ringer — in each case mean results of six experiments are displayed.

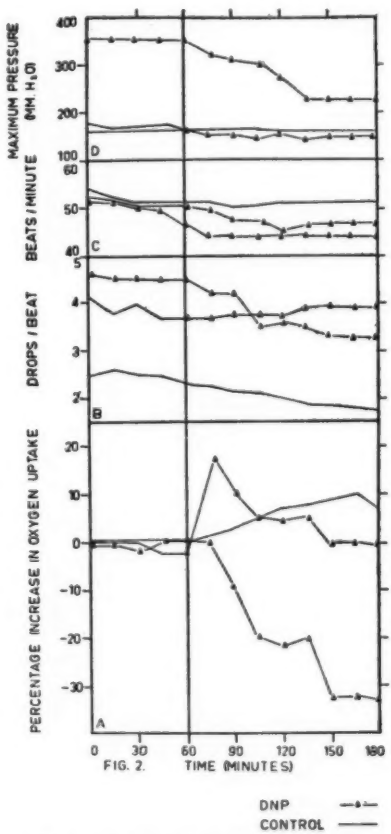

Fig. 2. Observations made following the addition of D.N.P. ($1{\cdot}09 \times 10^{-5}$M) to the preparation initially and after one hour's standard perfusion — mean results of four experiments.

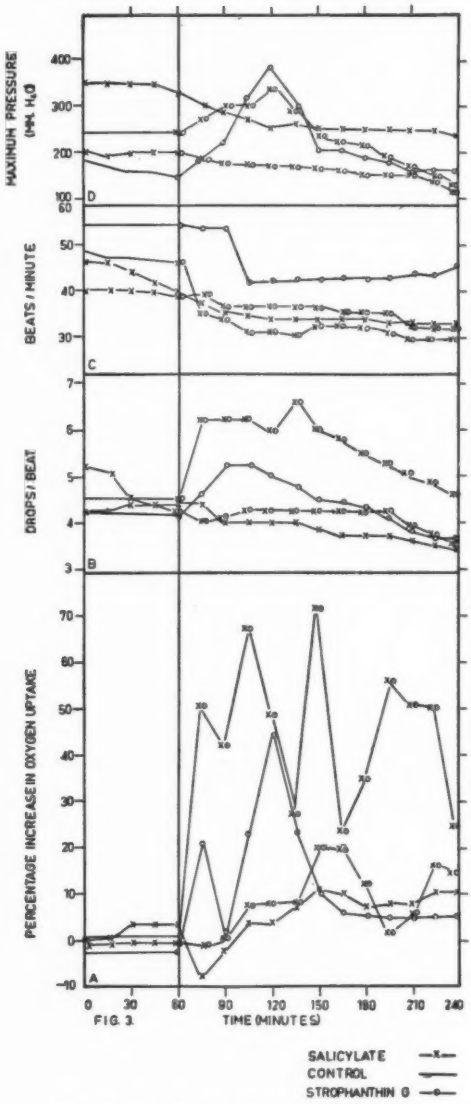

FIG. 3.

Fig. 3. Results showing the effect of sodium salicylate (5 mM) on the inotropic response of the heart to strophanthin-G (1×10^{-5})— in each case the mean results of six experiments are shown. Note that sodium salicylate, when initially added to the preparation, abolishes the positive inotropic response. The normal response was obtained when strophanthin-G and salicylate were added simultaneously.

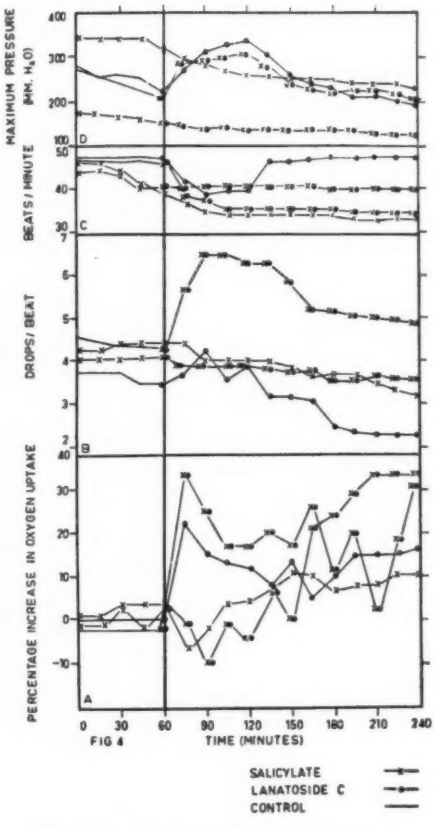

FIG 4

Fig. 4. Results demonstrating the effect of sodium salicylate (5 mM) on the inotropic response of the heart to lanatoside-C (2×10^{-5}) — in each case means results of six experiments are displayed. The inotropic response was recorded only when lanatoside-C and salicylate were added simultaneously.

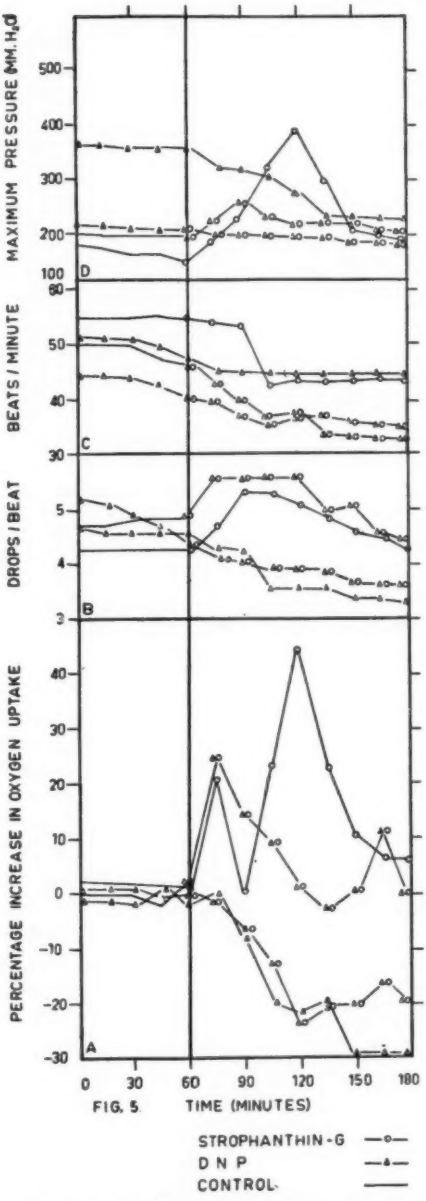

FIG. 5.

Fig. 5. Results showing effect of D.N.P. ($1{\cdot}09 \times 10^{-5}$M) on the inotropic response of the heart to strophanthin-G (1×10^{-5}) — mean results of four experiments. Note that pre-treatment with D.N.P. abolished the positive inotropic response.

control series above. In Fig. 1 B-D it is evident that the salicylate, at this concentration, did not markedly impair cardiac activity. The oxidative metabolism in these salicylate-treated hearts, as indicated by the rate of oxygen uptake, differed from that of the control series in that a small rise apparent during the first hour's perfusion was followed by a secondary fall throughout the second hour with a subsequent return to the control pattern—Fig. 1 A.

The addition of salicylate to the hearts after one hour's control perfusion in standard Ringer resulted in an increased cardiac output associated with an immediate and marked increase in oxygen uptake which was followed by a fall ninety minutes later. A very slight rise in maximum aortic pressure followed immediately by a decline was consistently noted after addition of the salicylate; no change in the beat rate was recorded.

D.N.P., when added at the commencement of the perfusion to give a final concentration $1{\cdot}09 \times 10^{-5}$M, elicited no marked spontaneous changes in beat rate or stroke volume; there was a gradual decline in maximum aortic pressure. A lowered oxygen utilization rate was apparent after two hours perfusion in the D.N.P. enriched perfusate. These results are summarized in Fig. 2 A-D. In Fig. 2 D there is some suggestion of reduced cardiac output associated with the depressed oxygen consumption referred to above.

When D.N.P. was added to the perfusate after one hour's standard perfusion in normal Ringer, an immediate stimulation of oxygen utilization was recorded (Fig. 2 A), associated

with a slow decline in the beat rate but no change in maximum aortic pressure or cardiac output—Fig. 2 B-D.

These results would seem to indicate that D.N.P. and salicylate, when used in these concentrations, elicit similar responses from the isolated heart, the main difference being the increased cardiac output produced by the salicylate. Continued perfusion in D.N.P. (one hundred and sixty minutes) resulted in a greatly depressed rate of oxygen utilization not shown by salicylate at this concentration.

In Fig. 3 A-D and 4 A-D the changed response of this heart preparation to the glycosides, strophanthin-G and lanatoside-C, in the presence of sodium salicylate (5 mM final concentration) is summarized and compared with the normal response of hearts to these glycosides. This normal response was a marked and sustained increase in cardiac output, maximum aortic pressure, oxygen uptake and decreased beat rate apparent thirty minutes after the addition of the drugs. In those preparations in which either of the glycosides were added together with sodium salicylate the normal inotropic response was recorded; there appeared to be some summation in the case of increased cardiac output and oxidative metabolism.

However, when the hearts were pre-treated for one hour and forty minutes with salicylate prior to the administration of the glycosides, no vestige of the usual inotropic response was recorded. As is shown in Figs. 3 and 4, the pattern of response in this last series is essentially that of the control preparations in which salicylate enriched Ringer was used throughout.

D.N.P., when added together with strophanthin-G did not interfere with the normal inotropic response — see Fig. 5 A-D. However, when strophanthin-G was added to preparations pre-treated for one hour and forty minutes with D.N.P., no inotropic response was recorded, all aspects of the response being completely abolished as was true for the salicylate series above.

DISCUSSION.

The perfused, spontaneously beating hypodynamic toad's heart has provided a convenient preparation for the comparative study of the cardiac changes evoked by the administration of the metabolic inhibitors D.N.P. and sodium salicylate. The concentration of sodium salicylate used lies within the therapeutic dose range recorded by Dorfman *et al.* (1947) and the concentration of D.N.P. was less than that required for the complete depression of oxidative phosphorylation (Ellis, 1952).

The immediate and marked increase in oxygen utilization recorded following the addition of sodium salicylate to this preparation supports the results of other workers using a variety of test methods. Smith and Jeffrey (1956) using rat diaphragm, Sproull (1954) using mouse liver slice, Fishgold *et al.* (1951) using rat brain preparations have all recorded augmented oxidative metabolism

following the addition of salicylate at the above concentration. Similarly, the metabolic actions of D.N.P. known from studies of various tissues *in vitro* include increased oxygen utilization — Lee (1954), Ehrenfest and Ronzoni (1933). Prolonged perfusion in Ringer containing D.N.P. apparently depressed oxidative metabolism subsequent to the initial stimulation. Recently Smith and Jeffrey (1956) have demonstrated that salicylate, in the above concentration, decreased the level of creatine phosphate and A.T.P. in rat diaphragm studies, suggesting that salicylate, like D.N.P., may cause a defect in energy production by interference with oxidative metabolism. This action has been well established in the case of D.N.P. by the work of Loomis and Lipman (1948), and by Cross *et al.* (1949). Other similarities between the action of these two drugs includes the fact that both are known to be glycogenolytic — Lutwak-Mann (1942), Magne *et al.* (1933).

Hitherto, all studies have been made upon tissue slice or homogenate. The results reported in this present study indicate that the intact organ does show a similar metabolic pattern of response to the two drugs, and that this response is clearly comparable with that displayed by the tissue slice and homogenate studies.

The rôle played by the higher energy phosphate compounds in the response of the hypodynamic heart to the digitalis glycosides is difficult to assess. Previous studies carried out in an endeavour to clarify this problem have used D.N.P. to limit the production of high energy phosphate bonds.

Both Ellis (1953) and Wollenberger and Karsh (1952) reported complete inhibition of the positive inotropic action of the digitalis compounds in the presence of D.N.P. However, Gruhzit and Farah (1954) reported that the cardiotonic effect of ouabain depended upon the severity of the D.N.P. poisoning, thereby supporting the results of Taeshler *et al.* (1953) and Rothlin *et al.* (1955) who were able to obtain a positive inotropic response in the dog heart-lung preparation provided that the failure induced by D.N.P. was mild.

The experiments in this series support the results of the latter workers, since when strophanthin-G or lanatoside-C was added with either D.N.P. or salicylate a normal inotropic response as expressed by stimulated oxidative metabolism, maximum aortic pressure, cardiac output and lowered beat rate was always recorded. However, in those hearts which had been previously perfused with D.N.P. or salicylate Ringer, thereby reducing the A.T.P. and phosphocreatine supply, no inotropic response was recorded. Apparently in those preparations in which the glycosides and inhibitors were added together, the phosphocreatine and adenosine triphosphate levels remained sufficiently high to allow the positive inotropic response to be manifested. It is interesting to note that the increased oxidative metabolism associated with the action of the cardiac glycosides was completely suppressed in the salicylate and D.N.P. treated hearts, indicating some correlation between the previously reported increased oxygen consumption and the positive inotropic response.

Since Greiner (1952) found normal phosphocreatine levels to be associated with reduced A.T.P. in hypodynamic hearts and was able to restore the depleted A.T.P. level to normal by the administration of glycosides, it would appear that the reduced adenosine nucleotide level following perfusion with D.N.P. or salicylate was the factor which limited the response to the glycosides. Snellman and Gelotte (1950) have shown that "Digitotal" inhibited the deamination of a substance resembling adenosine; Rand *et al.* (1955) were able to potentiate the action of ouabain on the guinea pig heart by the injection of adenosine.

Whereas the modifying effect of D.N.P. upon the action of the cardiac glycosides has been investigated by several workers, the interference between these cardiac drugs and sodium salicylate has apparently not been previously studied.

SUMMARY.

The actions of sodium salicylate (5 mM final concentration) and of D.N.P. ($1{\cdot}09 \times 10^{-5}$ final concentration) on the isolated perfused toad's heart have been studied.

Both drugs caused marked stimulation of oxidative metabolism.

The inotropic action of the glycosides in the presence of these two metabolic inhibitors was studied. Pre-treatment of the hearts with D.N.P. or salicylate abolished the inotropic response.

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THE PERSISTENCE OF ANTIBODIES IN THE ABSENCE OF ANTIGENIC STIMULUS

by H. K. WARD

(From the New South Wales Red Cross Blood Transfusion Service, Sydney).

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One of the many problems associated with antigen-antibody reactions is the length of time during which the antibody can be detected in the serum of the immunized subject after the last antigenic stimulus. The persistence of antibody is important from both practical and theoretical points of view.

In public health practice we rely on immunization to protect the community if it is difficult or impossible to prevent exposure to the parasite. Since immunity depends in large measure on the specific antibody to the parasite it is clearly desirable to know how long on the average the antibody is present after immunization is completed. Is any generalization possible? Probably not until a number of different antibodies have been studied from this aspect. Unfortunately, a complication of such studies is that the parasite is often present in the environment and a chance contact (constituting another antigenic stimulus) may occur at any time during the long period of the investigation. In other words, the last antigenic stimulus would not be under proper control.

On the theoretical side, there is much controversy about the mechanism by which antibody continues to be produced months and years after the last apparent antigenic stimulus. It is known that the half-life of the antibody molecule and the cells which synthesize the antibody is measured in days only. Does the foreign substance (the antigen) remain in the body indefinitely and continue to act as an antigenic stimulus? Or is the antigen eliminated quickly, but the antibody-forming cells continue indefinitely to transmit the capacity to synthesize antibody to one generation after another of these cells?

It is the purpose of this paper to give an account of the persistence of antibodies to the Rhesus antigen in human red cells. This system was chosen for a study of this nature because the only possible ways in which an Rh negative woman can be immunized against the Rhesus factor are (1) by transfusion of Rh positive blood, (2) by bearing an Rh positive child. Both these stimuli can be checked, thus pin-pointing the last possible antigenic stimulus.

Materials and Methods.

In the records of the New South Wales Blood Transfusion Service, there are data of hundreds of women whose sera have been examined by the Rh Reference Laboratory during the past ten years and found or suspected to contain Rh antibodies. Letters were sent to

these women asking them to attend the Blood Transfusion Centre, where a specimen of blood was taken from the vein and at the same time their obstetrical and transfusion history was recorded. In all, specimens were obtained from 632 women. Ninety-five specimens were excluded either because the woman was pregnant at the time, or because the Rh type of the last child was unknown, or because the diagnosis of haemolytic disease and the presence of Rh antibodies was in doubt. Miscarriages at 3 months or less were ignored, but miscarriages after 3 months, if they occurred after the birth of the last Rh positive child, were accepted as a "last antigenic stimulus". Although few in number, this introduces an error, as the foetus may have been Rh negative.

The blood was allowed to clot, the serum drawn off and kept frozen until the Rh antibody was titrated.

Technique of estimating the titre of the Rh antibody. Each specimen of serum was titrated against D positive cells suspended in: (1) Saline solution for estimating the complete antibody content. To prepare the suspension one drop of fresh citrated D positive blood was added to 99 drops of saline solution. (2) 10 p.c. polyvidone solution for estimating the incomplete antibody content (Ward, 1953). To prepare the suspension, one drop of fresh citrated D positive blood was added to 99 drops of 10 p.c. polyvidone solution.

As it was impossible to titrate all these sera against suspensions of the same red cells, it was necessary to determine the experimental error of titrations against suspensions of different red cells. Accordingly, the same serum was titrated against suspensions of a number of different D positive red cells. The error was found to be no greater than one tube in doubling dilutions of the serum.

Estimation of the complete antibody. One drop of the undiluted serum was placed in the first agglutination tube and with a Pasteur pipette doubling dilutions of the serum, in saline solution, were made in agglutination tubes 2-12 with the same pipette. One drop of 1 p.c. suspensions of D positive red cells in saline was then added to each of the twelve tubes. The tubes were shaken to mix the contents and placed in a 37° C. incubator. At the end of an hour, the tubes were centrifuged at about 2,000 r.p.m. for one minute. The button of red cells was removed carefully with a Pasteur pipette, spread on a slide and examined for evidence of agglutination. The titre of the complete antibody was expressed as the reciprocal of the concentration of serum in the last tube of the series which showed agglutination under the low power of the microscope.

Estimation of the incomplete antibody. One drop of the undiluted serum was placed in the first agglutination tube, and doubling dilutions of the serum in saline solution were made in agglutination tubes 2-12. One drop of 1 p.c. suspension of D positive red cells in 10 p.c. polyvidone solution was then added to each of the 12 tubes. The tubes were shaken to mix the contents and left at room temperature. At the end of an hour, the tubes were centrifuged at about 2,000 r.p.m. for one minute. Each tube was then rolled gently between the palms of the hands until the button of cells disappeared. The contents of the tube were then tipped on to a glass slide and examined for evidence of agglutination. The titre of the incomplete antibody was expressed as the reciprocal of the concentration of serum in the last tube of the series which showed agglutination under the low power of the microscope.

RESULTS.

Complete Antibody.

The results of the titration of the complete antibody are shown in Table 1, the sera being grouped according to the intervals which had elapsed since the last possible antigenic stimulus. For the first two years the sera are grouped at

six-monthly intervals in order to demonstrate the comparatively rapid fall in the titre of the complete antibody. For the third, fourth, fifth and sixth years after the last antigenic stimulus the sera were grouped at yearly intervals. After six years the number of sera was too small for the mean titre to be valuable. In Fig. 1 the mean titre of the various groups is plotted against the time that has elapsed since the last antigenic stimulus.

TABLE 1.

The titre of complete Rh antibodies.

Elapsed period after last antigenic stimulus	Number of sera with titre of													Total number of sera	Mean titre
	0	2	4	8	16	32	64	128	256	512	1024	2048	4096		
0-6 months	23	0	2		0	4	2	2	0	3	0	1	1	38	215·8
7-12 months	41	1	1	5	2	4	8	3	1	2	1	0	0	69	49·3
13-18 months	29	1	3	0	3	3	2	2	0	0	0	0	0	43	19·6
19-24 months	39	0	3	1	6	1	3	3	1	0	0	0	0	57	17·2
25-36 months	76	3	1	3	4	6	3	1	0	0	0	0	0	97	6·3
37-48 months	61	1	4	1	1	3	4	0	2	0	0	0	0	77	11·7
49-60 months	40	2	3	0	0	1	2	0	1	0	0	0	0	49	8·8
61-72 months	39	2	1	0	3	0	0	0	0	0	0	0	0	45	1·2

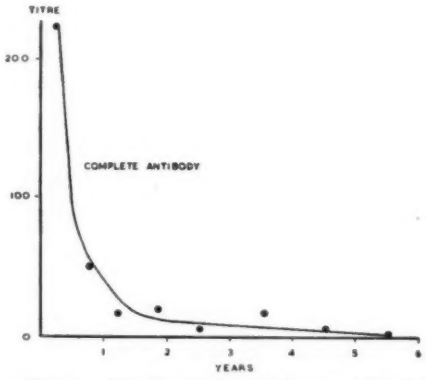

Fig. 1. The mean titre of the complete Rh antibody in immunized Rh negative women at various intervals after the last antigenic stimulus.

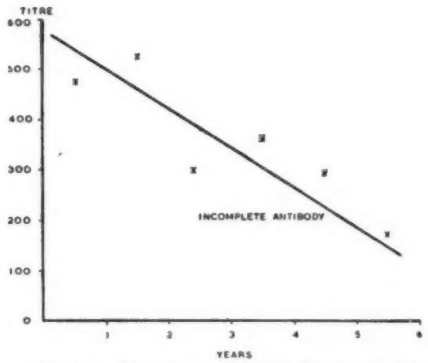

Fig. 2. The mean titre of the incomplete Rh antibody in immunized Rh negative women at various intervals after the last antigenic stimulus.

It will be seen from Table 1 that the majority of the subjects had no complete antibody in their serum at the time of testing. Fig. 1 shows that the mean titre of the complete antibody fell rapidly during the first eighteen months after the last stimulus, although in a few subjects the antibody persisted for some years.

Incomplete Antibody.

The results of the titration of the incomplete antibody are shown in Table 2, the sera being grouped according to the interval which has elapsed since the last antigenic stimulus. The sera are all grouped at twelve monthly intervals, as the fall in the titre of the incomplete antibody was much more gradual than was the case with the complete antibody.

TABLE 2.

The titre of incomplete Rh antibodies.

Elapsed period after last antigenic stimulus	Number of sera with titre of													Total number of sera	Mean titre
	0	2	4	8	16	32	64	128	256	512	1024	2048	4096		
0-12 months	2	5	7	2	2	2	5	16	24	19	18	5	1	108	473·6
13-24 months	3	2	5	1	3	4	11	13	19	17	18	1	4	101	524·8
25-36 months	8	3	5	6	4	1	10	13	24	15	5	2	1	97	305·3
37-48 months	3	1	6	4	1	3	11	6	17	17	6	2	1	78	372·2
49-60 months	2	2	1	1	4	2	10	5	11	5	4	2	0	49	305·9
61-72 months	0	1	3	4	0	4	8	9	10	5	1	0	0	45	177·1

It will be seen from Table 2 that the incomplete antibody had disappeared from a few of the sera but the majority had titres ranging from 64 to 512. Fig. 2 shows that the mean titre of the incomplete antibody after the last antigenic stimulus fell slowly, and even after six years the mean titre was still high.

DISCUSSION.

It is clear from these results that the two antibodies—complete and incomplete—produced as a result of Rh immunization behave differently.

Complete Rh antibodies. The complete antibody disappears comparatively quickly. In Fig. 1 the slope of the curve in the first year after the last antigenic stimulus would suggest the disappearance of this antibody in all subjects after two years. However, the complete antibody did persist in a few individuals for some years. The absence of this antibody in so many of these women raises some doubt whether it was ever formed in them. Diamond (1947) believes that generally the complete antibody is first formed and is later replaced by

incomplete antibody, especially with succeeding pregnancies. On the other hand, Waller (1949) found that five out of ten Rh negative volunteers who were deliberately immunized with Rh positive blood never developed the complete antibody.

Incomplete Rh antibodies. The incomplete antibody persists for many years. In Fig. 2 the curve of the mean titre shows a gradual decline and even six years after the last antigenic stimulus the mean titre is still high. The incomplete antibody was present in all the sera which were tested nine and ten years after the last stimulus, and several workers have detected it more than twenty years later. It would probably be fair to say that the incomplete antibody persists indefinitely in the absence of any further antigenic stimulus. As this antibody passes the placental barrier into the foetus, it means that once an Rh negative woman is immunized by Rh positive blood (whether from a transfusion or from a foetus) this incomplete Rh antibody threatens the health or life of any subsequent Rh positive child she may conceive.

Apart from the incomplete Rh antibody, comparatively little work has been done on incomplete antibodies. They have been demonstrated by the Coombs technique with a few other antigens, notably with *Brucella abortus* (Wilson and Merrifield, 1951). It is known that the incomplete Rh antibody persists in the serum for many years, but it is unknown whether persistence is a feature of all types of incomplete antibody. Second attacks of many of the infectious diseases are uncommon and presumably this immunity depends in large part on the persistence of antibodies. Unfortunately there are difficulties in the study of the duration and nature of these antibodies. In the first place the antigens (bacteria or viruses) are present in the environment, and one is always in doubt whether or not the antibodies have been reinforced by exposure to antigenic stimuli subsequent to the first attack of the disease. In the second place it is not nearly as easy to determine whether the antibody is complete or incomplete as it is in the case of the Rh antibodies. In many of the virus diseases all that is known is that the antibody neutralizes the virus.

The Persistence of Incomplete Rh Antibody. It is clear from this study that the incomplete Rh antibody is produced continuously and indefinitely in the absence of any antigenic stimulus subsequent to the original exposure to the Rh antigen. The life of the antibody molecule and of the antibody-producing cells is measured in days, but the presence of the antibody in the immunized subject is measured in years. How is this persistence of the antibody to be explained, not only the incomplete Rh antibody, but antibodies in general? There are two schools of thought on this question.

A. *Theory that antigen persists.*

The proponents of this theory believe that persistence of antibody can be explained by the antigen persisting in the body even though demonstration of the presence of the antigen is fraught with difficulty. Some confirmation of

this hypothesis has been provided by Coons *et al.* (1950) who by means of a fluorescent antibody demonstrated the presence of the antigen (in this case a pneumococcal carbohydrate) in the cells of the immunized animal months after the injection of the antigen. However, the pneumococcal carbohydrate may be a unique kind of antigen, for as Heidelberger (1953) has pointed out, this carbohydrate is most resistant to enzymic attack, and would not be eliminated from the body by breakdown into simpler substances.

Nevertheless, there are other examples of a persisting antigen. Burnet and Lush (1939) believe that the virus of herpes simplex causes a vesicular stomatitis usually in the one to three years age period, and then persists in some of the epithelial cells throughout life. Recurrent attacks of herpes labialis occur in some subjects. Neutralizing antibodies can be demonstrated in the serum of these individuals as well as in a large percentage of the adult population.

Zinsser (1934) pointed out that over 90 p.c. of patients suffering from Brill's disease on the east coast of America were migrants from eastern Europe some years previously. The rickettsia isolated from these patients were *R. prowazeki,* the organism of European typhus. This finding in Brill's disease was confirmed by Murray and Snyder (1951), and by Steel and Lawy (1956). Zinsser suggested that the most probable explanation was the persistence of *R. prowazeki* for many years in these patients with a subsequent relapse which was diagnosed as Brill's disease. This explanation has been strengthened by Price (1955) who isolated *R. prowazeki* from the lymph nodes of two subjects, not suffering from Brill's disease, but showing a low complement fixation titre against *R. prowazeki.* These individuals had come from Russia twenty years previously.

The difficulty that can be encountered in demonstrating the presence of a virus in the body is illustrated by experience with the virus causing papillomata in rabbits. This virus occurs naturally in the cotton-tail rabbit and can be recovered easily from the papillomata. While the virus causes papillomata in the domestic rabbit, the virus is not easily recovered from these animals. However, if rabbits are immunized with tumour tissue from domestic rabbits, virus neutralizing antibodies are formed showing that the virus is present in a masked form (Friedewald and Kidd, 1944).

The examples quoted can hardly be considered an impressive body of evidence in favour of the general persistence of antigens after the original infection, and where the antibody can be demonstrated for many years. Summing up the evidence for the persistence of antigen to explain the presence of persisting antibody, one could say "possible but not proven".

B. *Theory of transmissible alteration in antibody-forming cells.*

The other school of thought, associated with the names of its authors, Burnet and Fenner (1949), postulates a transmissible alteration in antibody-

forming cells as a result of the original contact with the antigen. This hypothesis does not necessitate the continuing presence of the antigen, as the alteration which produced the antibody globulin would be transmitted indefinitely to the succeeding generations of cells, presumably during the lifetime of the host. The alteration would not be transmitted to the next generation of the host, since it affects somatic cells and not germ cells.

This hypothesis has been advanced on the analogy of the production of adaptive enzymes. A cell which normally does not produce a certain enzyme can be trained to produce the enzyme if the cell is put in contact with the enzyme's substrate. In other words, cells have a potentiality to produce certain products which normally are not part of their activities. This product might be an enzyme or it might be an altered globulin, depending on the particular cell. This activated potentiality would then be transmitted to subsequent generations of cells although the original substrate or antigen is withdrawn.

This is an attractive hypothesis, but like the other explanation of a persisting antigen, it remains a hypothesis.

Heidelberger's observations.

Heidelberger (1953) has made an interesting contribution to this problem. He compares antibody production to a protein antigen, such as diphtheria toxoid, with antibody production to a carbohydrate antigen such as pneumococcal carbohydrate. Heidelberger points out (i) that the antitoxin produced in response to the toxoid fades out fairly rapidly, whereas the pneumococcal antibody produced in response to the carbohydrate tends to persist, (ii) that the toxoid is a protein and is liable to enzyme attack and presumably to breakdown and elimination, whereas the carbohydrate is resistant to enzyme attack and has been found unaltered in the cells months after infection, (iii) that on further stimulation with the appropriate antigen, say a year after the original immunization, the antitoxin level rises sharply from zero or near zero while the level of the pneumococcal antibody, still well above zero, shows no change as a result of further stimulation.

There is some analogy between Heidelberger's observations and our own findings with the Rh antibodies, the complete Rh antibody disappearing fairly rapidly and the incomplete Rh antibody persisting indefinitely. It is tempting to put forward the hypothesis (i) that the complete Rh antigen is a protein to which the complete antibody is formed in the first place, this antibody disappearing when the protein antigen is broken down by enzymic attack; (ii) that the protein antigen is not completely broken down, but an antigenic fraction, resistant to further degradation by enzymes, persists in the tissues and stimulates the formation of the persisting incomplete Rh antibody. Admittedly there is no proof whatever of this hypothesis. If there is an antigenic Rh haptene analogous to the pneumococcal haptene it has not been demonstrated.

SUMMARY.

Five hundred and thirty seven immunized Rh negative women were bled and their sera examined for their content of complete and incomplete Rh antibodies.

At the time of bleeding, the obstetrical history was taken and the interval calculated between the last antigenic stimulus and the date of bleeding. The interval varied between less than one month and twelve years.

When the mean antibody titre was plotted against the interval since the last antigenic stimulus, it was found (a) that the titre of the complete antibody, with a few exceptions, fell rapidly; and (b) that the titre of the incomplete antibody fell slowly over the years.

Two explanations of the persistence of antibodies are discussed briefly.

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